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(57) Abstract

The present invention relates to a cDNA coding for a novel protein which belongs to C6 β–chemokines and attracts subsets of peripheral blood leukocytes, and a process for preparing the said protein by employing expression vector therefor. Open reading frame of the Lkn–1 cDNA encodes 113 amino acids containing a signal peptide of 21 amino acids, and molecular weight of mature protein consisting of 92 amino acids among them is supposed to be 10,162 dalton. A recombinant Lkn–1 which was expressed in *E. coli* or insect cell employing the said Lkn–1 cDNA and purified, inhibited colony formation and cell proliferation significantly, attracted neutrophils, monocytes and lymphocytes to cause chemotaxis, and bound to CCR1 and CCR3 receptors. Accordingly, it was determined that the recombinant Lkn–1 protein can be used as a potential drug for antibody production, the treatment during HIV–1 infection, the protection of bone marrow stem cells during chemotherapy or radiotherapy and the inhibition of leukemia, etc.

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A cDNA ENCODING C6 β-CHEMOKINE LEUKOTACTIN-1(Lkn-1) ISOLATED FROM HUMAN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a cDNA encoding a novel C6 β-chemokine isolated from human body and a process for preparing the C6 β-chemokine, more specifically, to a cDNA coding for a novel protein which belongs to C6 β-chemokines and attracts subsets of peripheral blood leukocytes, a process for preparing the said protein by employing expression vector therefor, and pharmaceutical application of the said protein.

Description of the Prior Art

Chemokines are a family of small cytokines consisting of basic proteins of a low molecular weight, have four cysteines commonly, which are classified into four subfamilies of CXC(α), CC(β), C(γ) and CX_3C depending on the position of the first and the second cysteines, i.e., whether they lie adjacent or an amino acid intervenes between the two cysteines(see: Baggiolini, M. and Dahinden, C.A., Immunol. Today, 15:127(1994); Kelner, S.G. et al., Science, 266:1395(1994); Bazan, J.F. et al., Nature, 385:640(1997)). Genes of chemokine subfamilies locate on a same chromosome in a cluster, for example, α-chemokine genes locate on the human chromosome 4q12-21 and β-chemokine genes exist on the human chromosome 17q11-32 and the mouse chromosome 11.

Chemokines have biological activities such as HIV-

inhibitory action, immunoregulatory action, leukocyte migration and inhibitory action against division of hematopoietic stem cells(see: Cocchi, F. et al., Science, 270:1811(1995); Wolpe, S.D. et al., J. Exp. Med., 167:570(1988); Graham, G.J. et al., Nature, 344:442(1990); Broxmeyer, H.E. et al., Blood, 76:1110(1990); Youn, B.-S. et al., J. Immunol., 155:2661-2667(1995)).

Also, chemokines bind to transmembrane domain G protein-coupled receptors to activate leukocytes and some of the receptors are also used as coreceptors during HIV-1 infection(see: Oh, K.-O. et al., J. Immunol., 147:2978(1991); Alkabatih, G. et al., Science, 272:1955(1996)). For example, among 8 subtypes of β-chemokine(CC chemokine) receptor, i.e., CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7 and CCR8, four subtypes of CCR4, CCR6, CCR7 and CCR8 show a high affinity to one substance while CCR1, CCR2, CCR3 and CCR5 have a binding affinity to various chemokines.

So far, nine chemokines belonging to β-chemokine subfamily have been known in the art(see: Wilson, S.D. et al., J. Exp. Med., 171:1301(1990); Modi, W.S. et al., Hum. Genet., 84:185(1990)). Among them, a murine MRP-1("mMRP-1", MIP(macrophage inflammatory protein)-related protein-1 or C10)(see: Orlofsky, A. et al., Cell Regul., 2:403(1991)) and a murine MRP-2("mMRP-2")(see: Youn, B.-S. et al., J. Immunol., 155:2661(1995)) are distinguished from the rest of β-chemokines in that they have two extra cysteine residues, thereby forming the third disulfide bond and their N-terminal regions are very long. Based on the previous findings, they are classified into C6 β-chemokines.

Under the circumstances, there are strong reasons for exploring and developing novel human MRPs, since human MRPs may be employed as a potential drug for the treatment of HIV-1 infection or for the protection of bone marrow stem cells

in the course of chemotherapy or radiotherapy.

SUMMARY OF THE INVENTION

The present inventors have made an effort to isolate MRP genes from various human cell lines. As a result, the inventors isolated a novel cDNA of MRP belonging to C6 β-chemokines from a human monocytic cell line and determined its nucleotide sequence and amino acid sequence deduced therefrom. Further, they successfully expressed the said MRP cDNA in recombinant E. coli or insect cell, and discovered that: the expressed recombinant protein inhibited colony formation and proliferation of myeloid stem cell and progenitor cell in vivo and in vitro as well, and attracts subsets of peripheral blood leukocytes (lymphocyte, monocyte and neutrophil) in chemotactic fashion. Hereinafter, the said MRP cDNA isolated from human body and the recombinant MRP are referred to as "Lkn-1(leukotactin-1) cDNA" and "recombinant Lkn-1", respectively.

The first object of the invention is, therefore, to provide a novel cDNA encoding C6 β-chemokine(Lkn-1) and an amino acid sequence deduced therefrom.

The second object of the invention is to provide an expression vector comprising the said Lkn-1 cDNA and a recombinant microorganism transformed with the vector.

The third object of the invention is to provide a process for preparing a recombinant Lkn-1 from the said microorganism.

The fourth object of the invention is to provide a method for protecting myeloid cells from cytotoxic anti-cancer drugs or radiation using the recombinant Lkn-1.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1(A) shows a nucleotide sequence(SEQ ID NO:1) of a DNA fragment of 202bp and an amino acid sequence deduced therefrom(SEQ ID NO:2).

Figure 1(B) shows a comparison of the amino acid sequence(SEQ ID NO:2) of Lkn-1 with the 87th to 110th amino acid sequence(SEQ ID NO:3) of mMRP-2.

Figure 2 is a photograph showing a result of RT-PCR(reverse transcriptase-polymerase chain reaction) of total RNA isolated from various human cell lines.

Figure 3 shows a nucleotide sequence(SEQ ID NO:4) of a Lkn-1 cDNA and an amino acid sequence deduced therefrom(SEQ ID NO:5).

Figure 4 shows a comparison of an amino acid sequence of mature Lkn-1(SEQ ID NO:7) with those of mMRP-1(SEQ ID NO:8), mMRP-2(SEQ ID NO:9), hMIP-1α(SEQ ID NO:10), mMIP-1α(SEQ ID NO:11) and hMIP-1β(SEQ ID NO:12).

Figure 5(A) is a graph which shows effect of Lkn-1 on the colony formation of myeloid cells in bone marrow.

Figure 5(B) is a graph which shows effect of Lkn-1 on the colony formation of myeloid cells in spleen.

Figure 5(C) is a graph which shows effect of Lkn-1 on the proliferation rate of myeloid cells in bone marrow.

Figure 5(D) is a graph which shows effect of Lkn-1 on the proliferation rate of myeloid cells in spleen.

Figure 6(A) is a graph which shows dose-dependent suppressive effect of Lkn-1 on the proliferation rate of myeloid cells in bone marrow.

Figure 6(B) is a graph which shows dose-dependent suppressive effect of Lkn-1 on the proliferation rate of myeloid cells in spleen.

Figure 7(A) is a graph which shows time-dependent suppressive effect of Lkn-1 on the proliferation rate of CFU-GM in bone marrow.

Figure 7(B) is a graph which shows time-dependent suppressive effect of Lkn-1 on the proliferation rate of BFU-E in bone marrow.

Figure 7(C) is a graph which shows time-dependent suppressive effect of Lkn-1 on the proliferation rate of CFU-GEMM in bone marrow.

Figure 7(D) is a graph which shows time-dependent suppressive effect of Lkn-1 on the proliferation rate of CFU-GM in spleen.

Figure 7(E) is a graph which shows time-dependent suppressive effect of Lkn-1 on the proliferation rate of BFU-E in spleen.

Figure 7(F) is a graph which shows time-dependent suppressive effect of Lkn-1 on the proliferation rate of CFU-GEMM in spleen.

Figure 8(A) is a graph which shows a chemotaxis attracting lymphocytes by a recombinant Lkn-1 and RANTES(regulated on activation, normal T cell expressed and secreted).

Figure 8(B) is a graph which shows a chemotaxis attracting monocytes by a recombinant Lkn-1 and hMIP-1α.

Figure 8(C) is a graph which shows a chemotaxis attracting neutrophils by a recombinant Lkn-1 and IL-8.

Figure 9(A) is a graph showing relative fluorescence

measured in lymphocytes which are stimulated by a series of addition of RANTES and a recombinant Lkn-1.

Figure 9(B) is a graph showing relative fluorescence measured in lymphocytes which are stimulated by a series of addition of a recombinant Lkn-1 and RANTES.

Figure 9(C) is a graph showing relative fluorescence measured in monocytes which are stimulated by a series of addition of hMIP-1α and a recombinant Lkn-1.

Figure 9(D) is a graph showing relative fluorescence measured in monocytes which are stimulated by a series of addition of a recombinant Lkn-1 and hMIP-1α.

Figure 9(E) is a graph showing relative fluorescence measured in neutrophils which are stimulated by a series of addition of IL-8 and a recombinant Lkn-1.

Figure 9(F) is a graph showing relative fluorescence measured in neutrophils which are stimulated by a series of addition of a recombinant Lkn-1 and IL-8.

Figure 10(A) is a graph showing relative fluorescence measured in a HOS cell line expressing CCR1 which are stimulated by a series of addition of various agents.

Figure 10(B) is a graph showing relative fluorescence measured in a HOS cell line expressing CCR3 which are stimulated by a series of addition of various agents.

Figure 10(C) is a graph showing peak amplitude of calcium responses depending on concentrations of Lkn-1 and hMIP-1α.

Figure 10(D) is a graph showing peak amplitude of calcium responses depending on concentrations of Lkn-1, eotaxin and RANTES.

DETAILED DESCRIPTION OF THE INVENTION

To isolate the Lkn-1 gene from a human cell line, the present inventors first cloned an exon of the Lkn-1 gene, which may be used for the preparation of a probe. That is, human genomic DNA was digested with Hind III, separated on an agarose gel, and analyzed with southern blot by employing the ^{32}P-labelled mMRP-2 cDNA(see: Youn, B.S. et al., J. Immunol., 155:2661(1995)) as a probe to obtain a DNA fragment of 7.0kb which can be hybridized with mMRP-2. Then, to isolate an exon sequence of Lkn-1 gDNA from the HindIII-digested DNA fragment of 7.0kb, human genomic DNA was digested with HindIII completely, and separated on an agarose gel to isolate a DNA fragment of 7.0kb. The fragment thus obtained was inserted into a vector, and the vector thus obtained was introduced into a host cell. After transformation, a colony showing hybridization with the said mMRP-2 cDNA probe was selected. After a DNA fragment of 7.0kb was isolated from the colony and digested with AluI, a DNA fragment of 202bp which can be hybridized with mMRP-2 cDNA was cloned. And then, the nucleotide sequence of the fragment was determined, which

differentiated parts of intron and exon from the DNA fragment.

Then, in order to clone a novel Lkn-1 cDNA from a human cell line, Lkn-1 PCR primers permitting amplification of a 100bp-DNA fragment from the Lkn-1 exon sequence confirmed as above were prepared, and RT-PCR was carried out using total RNAs isolated from various human cell lines as a template, respectively, to select cell lines having Lkn-1 mRNA. One of the cell lines thus selected, a human monocytic THP-1 cell line activated by interleukin-4 (IL-4) was chosen and its cDNA library was prepared. On the other hand, RT-PCR was carried out using total RNA of the said THP-1 cell line as a template and the said Lkn-1 PCR primer to prepare a 100bp-DNA fragment of Lkn-1 exon. The cDNA library of a THP-1 cell line constructed as above was hybridized with a probe of the 100bp-DNA fragment of Lkn-1 exon. As a result, a Lkn-1 cDNA showing a positive reaction was obtained.

Determination of the nucleotide sequence of the said Lkn-1 cDNA and the deduced amino acid sequence revealed that the Lkn-1 cDNA is a novel one, open reading frame of the Lkn-1 cDNA encodes 113 amino acids containing a signal peptide of 21 amino acids, and the predicted molecular weight of mature Lkn-1 protein consisting of 92 amino acids is 10,162 dalton. Also, it was found that the Lkn-1 has no potential N-glycosylation site and belongs to C6 β-chemokine family having two cysteine residues as in mMRP-1 and mMRP-2, in addition to four cysteine residues commonly appeared in β-chemokine family.

The novel Lkn-1 cDNA thus cloned was inserted into an expression vector and introduced into a host cell such as E. coli or insect cell to express the recombinant Lkn-1.

On the other hand, myelosuppressive activity of the recombinant Lkn-1 was investigated for myeloid stem cell and progenitor cell derived from human bone marrow and spleen, which demonstrated that the recombinant Lkn-1 inhibited

colony formation and proliferation of the myeloid cells in a concentration- and time-dependent manner in vivo and in vitro as well. Further, it was observed that the recombinant Lkn-1 attracted human peripheral blood neutrophils, monocytes and lymphocytes strongly to cause chemotaxis, and induced calcium flux in the said cells. Particularly, it was found that the recombinant Lkn-1 binds to the RANTES and hMIP-1α receptors and does not bind to a IL-8 receptor (although it attracts neutrophils strongly to cause chemotaxis, like IL-8). Also, it was revealed that receptors of the recombinant Lkn-1 are CC chemokine receptor 1 (CCR1) and CCR3. The recombinant Lkn-1 was a stronger agonist at CCR1 than hMIP-1α or RANTES which had been already known to bind to CCR1, and it was a stronger agonist at CCR3 receptor than RANTES although it was a weaker agonist the receptor than eotaxin.

The recombinant Lkn-1 of the invention showing the characteristics mentioned above can be used for antibody production, the treatment during HIV-1 infection, the protection of bone marrow stem cells during chemotherapy or radiotherapy and the inhibition of leukemia, etc.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Cloning of exon of Lkn-1 genomic DNA

In order to clone a genomic DNA homologous to mMRP-2 from human, total human genomic DNA was digested with BamHI, EcoRI, Hind III, PstI, XbaI and XhoI, fractionated on a 1.0% agarose gel, and analyzed by southern blot employing a ^{32}P-labelled mMRP-2 cDNA as a probe to obtain a HindIII fragment of 7.0kb showing a positive signal.

In order to prepare sublibrary of the said HindIII fragment, 100Fg of human genomic DNA was digested with HindIII completely, and fractionated on a 1% agarose gel at a voltage of 20V for 16 hours. Then, a DNA fragment near 7.0kb was isolated employing Gene-clean kit (Bio 101, USA) and inserted into a HindIII-digested dephosphorylated pBluescript SK^+ vector (Stratagene, USA). Electro-max (GIBCO-BRL, USA) competent cell was transformed with the recombinant vector thus prepared by the aid of electroporation and cultured on a solid medium. About $2x10^5$ colonies formed was hybridized with the mMRP-2 probe. As a result, it was found that only one colony showed a positive signal of hybridization and contained the insert DNA of 7.0kb.

In order to isolate an exon sequence from the said genomic DNA fragment of 7.0kb, the pBluescript SK^+ vector containing the insert DNA of 7.0kb was isolated from the said colony showing a positive signal of hybridization, digested with AluI, fractionated on a 1.5% agarose gel, and analyzed by Southern blot to clone a DNA fragment of 202bp which was hybridized with a mMRP-2 cDNA.

A nucleotide sequence (SEQ ID NO:1) of the said DNA fragment of 202bp was determined to find intron and a part of exon translated into amino acids (see: Figure 1(A)). As a result, it was found that the amino acid sequence (SEQ ID NO:2) of the part of exon showed homology of 50% to that of mMRP-2 from the 87th to 110th amino acid and the second cysteine (indicated as "*") of two additional cysteines common in mMRP-1 and mMRP-2 was conserved in Lkn-1 (see: Figure 1(B)). In Figure 1(B), boxes showed amino acids conserved in Lkn-1 and mMRP-2.

Example 2: Cloning of a Lkn-1 cDNA

In order to clone a Lkn-1 cDNA from a human cell line, PCR primers permitting amplification of the Lkn-1 exon of

100bp, i.e., a forward primer of 5'-TTCCTCACCAAGAAGGGG-3'(SEQ ID NO:13) and a reverse primer of 5'-CTTTTTCATGCAATCCTG-3'(SEQ ID NO:14) were first prepared based on the amino acid sequence(SEQ ID NO:2) disclosed in Figure 1(B). Then, PCR was carried out using the 202bp-DNA fragment prepared in Example 1, total RNA of a human monocytic THP-1 cell line(ATCC TIB202) activated by 100Fg/ml of IL-4 for 24 hours, total RNA of a macrophage cell line U937(ATCC CTL1593) and total RNA of a HL-60 cell line(ATCC CCL240) as a template, respectively(see: Figure 2).

In Figure 2, lanes 1 to 4 show results of PCR using the 202bp-DNA fragment as a positive control, total RNA of the THP-1 cell line, total RNA of the U937 cell line and total RNA of the HL-60 cell line, respectively; and, lane M shows 100bp-ladder as a DNA size-marker. As shown in Figure 2, the THP-1 cell line activated by IL-4 produced Lkn-1 mRNA.

In order to prepare THP-1 cDNA library, human mRNA was isolated from the THP-1 cell line activated by IL-4, reverse-transcribed employing Time Saver cDNA kit(Pharmacia Biotech, USA) to give double stranded cDNA, and attached with BstXI adapter(Invitrogen, USA). Then, fractionation on an agarose gel was performed to isolate a cDNA of 0.5kb or more, which was inserted into a PRc/CMV(Invitrogen, USA) vector digested with BstXI to prepare cDNA library. The cDNA library was hybridized with the 100bp-DNA fragment of Lkn-1 exon amplified by PCR above as a probe. As a result, one cDNA clone showing a positive signal was isolated.

Example 3: Nucleotide sequencing of the Lkn-1 cDNA

Figure 3 shows a nucleotide sequence(SEQ ID NO:4) of the cDNA clone obtained in Example 2 and an amino acid sequence deduced therefrom(SEQ ID NO:5). In Figure 3, the underlined is a signal peptide; the sequence in a box is the amino acid sequence(SEQ ID NO:2) of Lkn-1 shown in Figure 1(B) which

was used as a probe during the screening of THP-1 cDNA library; and, (***) indicates a translation stop codon.

As shown in Figure 3, open reading frame of the Lkn-1 cDNA(SEQ ID NO:6) encodes 113 amino acids consisting of 21 amino acids forming a signal peptide and 92 amino acids forming a mature protein whose molecular weight is supposed to be 10,162 dalton. Also, it was found that there is no N-glycosylation site in the deduced amino acid sequence(SEQ ID NO:5) of Lkn-1.

Figure 4 shows comparison of an amino acid sequence of mature Lkn-1(SEQ ID NO:7) with those of mMRP-1(SEQ ID NO:8), mMRP-2(SEQ ID NO:9), hMIP-1α(SEQ ID NO:10)(see: Youn, B.S. et al., J. Immunol., 155:2661(1995)), mMIP-1α(SEQ ID NO:11)(see: Kwon, B.S. and Weissman, S.M., Proc. Natl. Acad. Sci., USA, 86:1963(1989)) and hMIP-1β(SEQ ID NO:12)(see: Sherry, B. et al., J. Exp. Med., 168:2251(1988)). In Figure 4, boxes and (•) indicate four conserved cysteine residues and two additional cysteine residues, respectively. As shown in Figure 4, it was revealed that: (1)Lkn-1 has a long N-terminal region before the first two cysteine residues of four cysteine residues common in β-chemokine family; and, (2)Lkn-2 belongs to C6 β-chemokine family having two cysteine residues further as in mMRP-1 and mMRP-2 besides four cysteine residues common in β-chemokine family. Moreover, it was found that the amino acid sequence(SEQ ID NO:7) of mature Lkn-1 shows homology of only about 43% to that of mMRP-1(SEQ ID NO:8) or mMRP-2(SEQ ID NO:9) and homology of 40% to that of hMIP-1α(SEQ ID NO:10) although Lkn-1 was isolated as a human counterpart of mMRP-2.

Example 4: Expression of a recombinant Lkn-1

Example 4-1: Expression of a recombinant Lkn-1 in E. coli

In order to express only a mature Lkn-1 protein without the putative signal peptide as a recombinant protein, PCR amplification of the open reading frame of the mature Lkn-1 was carried out using the Lkn-1 cDNA cloned in Example 2 as a template, the following PCR primers and Pfu polymerase(Stratagene, USA):

forward primer:
5'-CGAATTCCATATGCAGTTCACAAATGATGCAGAG-3'
(SEQ ID NO:15)

reverse primer:
5'-CGCCGCTCGAGTTGAGTAGGGCTTCAGC-3'
(SEQ ID NO:16)

The DNA fragment thus amplified was digested with NdeI/XhoI and cloned into a plasmid pET21a(Novagen, USA). The recombinant plasmid thus constructed was designated as pET21a-Lkn-1 and introduced into E. coli XL-1 Blue. The recombinant plasmid pET21a-Lkn-1 permits expression of a recombinant Lkn-1 which has one additional methionine residue in N-terminus and six additional histidines at C-terminus of a mature Lkn-1.

Transformant thus prepared was designated as 'Escherichia coli(XL-1 Blue) hMRP-2', and deposited with American Type Culture Collection(ATCC, Rockville, MD 20852, USA), an international depositary authority as deposition No. ATCC 98166 on September 10, 1996.

The said transformant was cultured to express Lkn-1, and inclusion bodies were obtained, dissolved in 20ml of denaturation buffer(6M guanidine-HCl, 20mM Tris-HCl, pH 7.9,

500mM NaCl, 4mM n-octylglucopyranoside) and centrifuged to obtain a supernatant. Then, chromatography using an activated Ni-column(Novagen, USA) and a heparin-agarose column(Pharmacia Fine Chemicals, USA) was carried out to purify a His-tagged recombinant Lkn-1. Electrophoresis revealed that a molecular weight of a recombinant Lkn-1 is about 12kDa.

Example 4-2: Expression of a recombinant Lkn-1 in an insect cell

In order to express a recombinant Lkn-1 containing a signal peptide, a Lkn-1 cDNA containing a signal peptide sequence was inserted with a PstI restriction site at N-terminus and a XbaI restriction site at C-terminus, and amplified by PCR. Then, the PCR product thus amplified was isolated and inserted into a PVL 1392 vector(Invitrogen, USA) digested with PstI/XbaI to construct a recombinant plasmid PVL 1392-Lkn-1. And then, a Sf-21 insect cell was transfected with both the said recombinant plasmid and AcNPV(Autographa california nuclear polyhedrosis baculovirus) to transfer the Lkn-1 cDNA in the said recombinant plasmid to the AcNPV. AcNPV-Lkn-1 viral plaque was isolated based on the characteristics that the virion is occlusion-negative, and grown in the Sf-21 insect cell in a serum-free Ex-Cell 400 medium(JRH Biosciences, USA) for later use.

A recombinant Lkn-1 was expressed in a High five cell line(Invitrogen, USA) cultured in a Ex-cell 400 medium, and the Lkn-1 thus expressed was purified using a HiTrap-Heparin column(Pharmacia Biotech., USA) and a HiTrap-SP column(Pharmacia Biotech., USA). Western blot analysis revealed that a molecular weight of the recombinant Lkn-1 analyzed immediately after purification is about 12kDa.

Example 5: Myelosuppressive activity of recombinant Lkn-1

Example 5-1: Inhibition of colony formation of bone marrow cells by the recombinant Lkn-1 in vitro

Since some of the β-chemokines have a myelosuppressive activity in vitro, an effect of the recombinant Lkn-1 purified in Example 4-1 on the colony formation by myeloid progenitor cell present in human bone marrow was investigated. That is, in order to form colony of bone marrow cells by CFU-GM(colony forming unit-granulocyte-macrophage), human bone marrow cells of low density obtained by centrifugation employing Ficoll-Hypaque gradient(1.070gm/cm^3, Sigma Chemical Co., USA) were plated on a 0.3% agar culture medium containing 10% FBS in a concentration of 5x10^{4}cells/ml, and stimulated by rhGM-CSF(recombinant human granulocyte-macrophage-colony stimulating factor, 100U/ml, Immunex Corporation, USA) + rhSLF(recombinant human steel factor, 50ng/ml, Immunex Corporation, USA). On the other hand, in order to form colony of bone marrow cells by CFU-GM, BFU-E(burstforming unit-erythroid) and CFU-GEMM(colony forming unit-granulocyte-erythroid-macrophage-megakaryocyte), the said bone marrow cells were plated on a 1% methylcellulose culture medium containing 30% FBS in a concentration of 5x10^4 cells/ml, and stimulated by rhEPO(recombinant human erythropoietin, 1U/ml, Amgen Corporation, USA), rhIL-3(recombinant human interleukin-3, 100U/ml, Immunex Corporation, USA) or rhSLF.

After the stimulation, the cells were cultured in a BNP-210 incubator(Tabai ESPEC Corp., USA) under an environment of 5% CO_2 and 5% O_2, and number of colonies was counted after 14 days(see: Table 1). In this connection, the recombinant Lkn-1 was added in a concentration of 3-50ng/ml to a plate.

Table 1. Effect of the recombinant Lkn-1 on the colony formation of human bone marrow cells of low density

Sample	Concentra-tion	Agar		Methylcellulose		
		CFU-GM [GM-CSF][b]	CFU-GM [GM-CSF + SLF][b]	CFU-GM	BFU-E [EPO, SLF, IL-3][b]	CFU-GEMM
Control[a]		17±6	67±2	66±12	94±2	9±1
Recombi-nant Lkn-1	50ng/ml	16±4(-6)[c]	35±6(-48)[d]	25±3(-62)[d]	35±3(-63)[d]	5±1(-44)[d]
	25ng/ml	18±1(+6)	40±2(-40)[d]	33±4(-50)[d]	50±3(-47)[d]	6±1(-33)[d]
	6.25ng/ml	18±3(+6)	52±6(-22)[d]	45±6(-32)[d]	65±2(-31)[d]	7±2(-22)[d]
	3.125ng/ml	17±8(0)	63±5(-6)	61±10(-8)	95±6(+17)	10±1(+11)

a: Experimental group without addition of the recombinant Lkn-1 to the reaction solution
b: Growth factor used for the stimulation of colony formation
c: Level of change compared with the control (%)
d: Level of change compared with the control is significant($p<0.001$).

As can be seen in Table 1 above, the recombinant Lkn-1 inhibited colony formation by CFU-GM, BFU-E and CFU-GEMM significantly in a concentration-dependent manner. Level of the inhibition of colony formation was 22-63% compared with the control. On the other hand, it was found that the recombinant Lkn-1 cannot inhibit colony formation by CFU-GM stimulated by GM-CSF alone or by BFU-E stimulated by EPO alone, which demonstrates that the recombinant Lkn-1 has an inhibitory effect on immature progenitor cells which can be stimulated by various growth factors.

Example 5-2: Inhibition of proliferation of myeloid cells by the recombinant Lkn-1 in vivo

The biological activities of Lkn-1 were evaluated in vivo. That is, the purified Lkn-1 was intravenously injected into C3H/HeJ mice and absolute numbers of granulocyte macrophage(CFU-GM), erythroid(BFU-E) and multipotential progenitor cells(CFU-GEMM) and their proliferation rate in bone marrow and spleen were determined, respectively: C3H/HeJ mice were injected through the tail vein with either 0.1ml of sterile pyrogen-free saline or 8 μg of purified Lkn-1 diluted in sterile pyrogen-free saline. After 24h from the injection, myeloid cells of low density were prepared from bone marrow of femur and spleen of the mice analogously as in Example 5-1. Then, CFU-GM was plated on 0.3% agar culture medium, and stimulated by 10% PWM mouse spleen cell-conditioned medium. Similarly, BFU-E and CFU-GEMM were plated on 0.9% methylcellulose culture medium, respectively, and stimulated by 1 uint of rhEPO, 0.1mmole/L hemin and 1% PWM mouse spleen cell-conditioned medium. In this connection, the bone marrow and spleen cells were plated at respective concentrations of 7.5×10^4 and 1.0×10^6 cells/ml. After the stimulation, the cells were cultured under the same condition described in Example 5-1, and number of colonies was counted after 5 to 7 days of incubation(see: Figures. 5(A) and 5(B)). On the other hand, proliferation rates, i.e., cycling rates of CFU-GM, BFU-E and CFU-GEMM were determined as percentage of the cells in S-phase of cell cycle by mesuring the proportion of progenitors in DNA synthesis(i.e., S-phase of cell cycle) by the aid of specific activity(20Ci/mmol), tritiated thymidine(50 μCi/ml) kill technique, which is based on in vitro calculation of reduction in the number of colonies formed after pulse exposure of cells to hot tritiated thymidine for 20min as compared with a

comparable amount of cold thymidine(see: Figures. 5(C) and 5(D)). Figures 5(A) to 5(D) revealed that Lkn-1 rapidly decreased numbers of colonies by myeloid stem/progenitor cells and their proliferation rate(i.e., cell cycling rate) in the bone marrow and spleen. On the other hand, the nucleated cellularity in bone marrow, spleen and blood were assessed, and found to be not significantly affected as compared with control.

In order to investigate dose-dependency of these suppressive effects of Lkn-1, cycling rates of CFU-GM, BFU-E and CFU-GEMM were determined analogously as described above except for varying concentration of Lkn-1 from 0.1 to 20μg(see: Figures 6(A) and 6(B)). As can be seen in Figures 6(A) and 6(B), it was demonstrated that: CFU-GM, BFU-E and CFU-GEMM from the mice receiving 3 to 10μg of Lkn-1 were in a noncycling or slow cycling state in both marrow and spleen, while the cells from the mice receiving 0.1 to 1μg of Lkn-1 showed no change in their cycling rate except for splenic CFU-GEMM. The overall cell cycling rate was 80 to 90% decreased by Lkn-1, and BFU-E and CFU-GEMM appeared to be more sensitive to Lkn-1 than CFU-GM.

Further, in order to investigate time-dependency of these suppressive effects of Lkn-1, C3H/HeJ mice were injected with 8μg of Lkn-1, and the cell cycling status of CFU-GM, BFU-E and CFU-GEMM from bone marrow and spleen were examined respectively, at different time points(see: Figures 7(A) to 7(F)). As shown in Figures 7(A) to 7(F), the suppressive effect of Lkn-1 was time-dependent and reversible in bone marrow and spleen as well. That is, Lkn-1 placed the CFU-GM, BFU-E and CFU-GEMM in a noncycling or a slow cycling state within 12h and returned them to control level after 72h.

These dose- and time-dependent, and reversible suppressive effects of Lkn-1 in vivo on proliferation of

myeloid cells strongly suggested that: Lkn-1 has potential clinical use in protecting normal hematopoietic cells from cytotoxic anti-cancer drugs or radiation.

Example 6: Investigation of the recombinant Lkn-1 as a chemokine

In order to investigate whether the recombinant Lkn-1 may cause chemotaxis, peripheral blood mononuclear cells(PBMC) of a healthy man were obtained by centrifugation employing Ficoll-Hypaque gradient(1.077gm/cm^3). And then, monocytes were isolated from the PBMC thus obtained based on their attached activity onto the surface of plastic, and the said isolation step was repeated twice. In this connection, cells remained after the step for the monocyte isolation were obtained as lymphocytes. Purity of the monocytes and lymphocytes thus obtained was determined by microscopic examination of cytospin dyed with Diff-Quick(Baxter Scientific, USA). As a result, it was found that purity of the monocytes and lymphocytes was 90% and 88%, respectively.

Also, red blood cells were precipitated by using 3% Dextran T 500(Pharmacia Fine Chemicals, USA) and obtained by centrifugation employing Ficoll-Hypaque gradient. The red blood cells thus obtained were dissolved in a hypotonic solution to obtain human neutrophils. Purity of the neutrophils thus obtained was determined by morphology. As a result, it was found that the purity was 95%.

The monocytes and lymphocytes isolated above were suspended in RPMI 1640(Gibco, USA) containing 0.5% low endotoxin BSA(Sigma Chemical Co., USA) and 20mM Hepes in concentrations of $2x10^6$ cells/ml and $8x10^6$ cells/ml, respectively. The neutrophils were suspended in HBSS in a concentration of $1x10^6$ cells/ml.

Then, level of cell migration in a 48-well

microchamber(Neuroprobe, USA) was determined as followings. Lower wells of the microchamber were filled with only a buffer solution(control) or a buffer containing the recombinant Lkn-1, hMIP-1α (PeproTech., USA), RANTES(PeproTech., USA), IL-8(PeproTech., USA) or eotaxin(PeproTech., USA), and upper wells were filled with 50Fl of the said cell suspensions. A well was partitioned by a suitable filter without polyvinylpyrolidine to give a lower well and an upper well. When neutrophils and lymphocytes were used, a diameter of the filter pore was 3Fm. When monocytes were used, it was 5Fm. After the said microchamber was incubated at 37°C for 1 hour(for neutrophils), 2 hours(for monocytes) or 4 hours(for lymphocytes), the filter was separated from the chamber and washed. The cells on the filter were fixed and dyed with Diff-Quick. Thus, number of the cells was counted(see: Figures 8(A) to 8(C)).

In Figures 8(A) to 8(C), a value obtained by dividing number of migrating cells in an experimental group treated with a chemokine by number of migrating cells in a control was represented as a chemotactic index. Figure 8(A) is a graph which shows chemotaxis attracting lymphocytes by the recombinant Lkn-1 and RANTES. Figure 8(B) is a graph which shows chemotaxis attracting monocytes by the recombinant Lkn-1 and hMIP-1α. Figure 8(C) is a graph which shows chemotaxis attracting neutrophils by the recombinant Lkn-1 and IL-8.

As shown in Figures 8(A) to 8(C), it was revealed that the recombinant Lkn-1 is a strong chemokine attracting human peripheral blood neutrophils, monocytes and lymphocytes. Also, the recombinant Lkn-1 showed chemotaxis attracting neutrophils in a similar manner to IL-8(see: Figure 8(C)) and chemotaxis attracting monocytes in a similar manner to hMIP-1α(see: Figure 8(B)); and, it showed an improved chemotaxis attracting lymphocytes in a concentration-

dependent manner up to a concentration of 10Fg/ml of Lkn-1 although it showed lower level of chemotaxis than RANTES (see: Figure 8(A)).

Example 7: Analysis of calcium flux by the recombinant Lkn-1

Example 7-1: Analysis of calcium flux in lymphocytes, monocytes and neutrophils

It was investigated whether the recombinant Lkn-1 may bind to a receptor for activation of lymphocytes, monocytes and neutrophils to induce calcium efflux. Its competitive relationships with other agonists against the receptor was also examined. Receptor activation was determined by measuring a change in $[Ca^{2+}]$ in subsets(lymphocytes, monocytes and neutrophils) of peripheral blood leukocytes isolated using a MSIII fluorimeter(Photon Technology Internationl, USA). That is, cells were reacted with 2FM fura-2AM(Sigma Chemical Co., USA) at 37°C for 45 minutes, washed twice, and resuspended in HBSS(pH 7.4) containing 0.05% BSA in a concentration of $1x10^7$ cells/ml. 2ml of the cell suspension was added into a stirred, water-jacketed cuvette, and activated continuously at 340nm and 380nm at 37°C. Emitted fluorescence was measured at 510nm before and after addition of 25nM agonist(the recombinant Lkn-1, hMIP-1α, RANTES, IL-8 or eotaxin)(see: Figures 9(A) to 9(F)).

In Figures 9(A) to 9(F), relative fluorescence was expressed as a relative ratio of fluorescence activated at 340nm and 380nm. Figure 9(A) shows relative fluorescence measured in lymphocytes which are stimulated by a series of addition of RANTES and the recombinant Lkn-1; Figure 9(B) shows relative fluorescence measured in lymphocytes which are stimulated by a series of addition of the recombinant

Lkn-1 and RANTES; Figure 9(C) shows relative fluorescence measured in monocytes which are stimulated by a series of addition of hMIP-1α and the recombinant Lkn-1; Figure 9(D) shows relative fluorescence measured in monocytes which are stimulated by a series of addition of the recombinant Lkn-1 and hMIP-1α; Figure 9(E) shows relative fluorescence measured in neutrophils which are stimulated by a series of addition of IL-8 and the recombinant Lkn-1; Figure 9(F) shows relative fluorescence measured in neutrophils which are stimulated by a series of addition of the recombinant Lkn-1 and IL-8. As shown in Figures 9(A) to 9(F), it was found that the recombinant Lkn-1 induced calcium influx in lymphocytes, monocytes and neutrophils.

On the other hand, when G protein-coupled receptors were continuously exposed to ligands having the same binding site as the receptor signal within a short time, the said receptors were desensitized. Such a phenomenon occurred when the said cells expressing receptors were stimulated by the recombinant Lkn-1.

As shown in Figures 9(A) to 9(F), it was found that the recombinant Lkn-1 desensitized lymphocytes and monocytes completely when stimulation by RANTES and hMIP-1α followed(see: Figures 9(B) and 9(D)). Also, RANTES or hMIP-1α did not desensitize lymphocytes and monocytes completely when stimulation by the recombinant Lkn-1 followed(see: Figures 9(A) and 9(C)), which demonstrated that the recombinant Lkn-1 shares receptors of RANTES and hMIP-1α, and induces more calcium flux than RANTES and hMIP-1α. In addition, neutrophils were not desensitized by the recombinant Lkn-1 during a further stimulation by IL-8 although the recombinant Lkn-1 induced calcium flux in neutrophils(see: Figure 9(F)). Moreover, IL-8 did not desensitize neutrophils during a further stimulation by the

recombinant Lkn-1 (see: Figure 9(E)). Therefore, it was clearly demonstrated that the recombinant Lkn-1 does not share IL-8 receptors although it is a strong factor to induce calcium flux in neutrophils like IL-8.

Example 7-2: Analysis of calcium flux in a cell line expressing CC chemokine receptors

It was investigated whether the recombinant Lkn-1 can activate a cell line expressing CC or CXC chemokine receptors because it was found in Example 7-1 that the recombinant Lkn-1 activated receptors which can be activated by RANTES and hMIP-1α. Analysis was carried out in the same manner as in Example 7-1 except for the use of a HOS cell line expressing recombinant CCR1, CCR2B, CCR3, CCR4, CCR5 or CXCR4 (see: AIDS Research and Reference Reagent Program of NIH, USA) instead of the use of subsets of leukocytes isolated. It has been known that hMIP-1α binds to both CCR1 and CCR5, and RANTES binds to CCR1, CCR3 and CCR5.

Figure 10(A) shows relative fluorescence measured in a HOS cell line expressing CCR1 which are stimulated by a series of addition of various agents, and Figure 10(B) shows relative fluorescence measured in a HOS cell line expressing CCR3 which are stimulated by a series of addition of various agents.

As shown in Figures 10(A) and 10(B), it was revealed that the recombinant Lkn-1 strongly induced calcium flux in a HOS cell line expressing CCR1 or CCR3. However, calcium flux by the recombinant Lkn-1 in HOS cell lines expressing other receptors was not observed.

Also, as shown in Figure 10(A), CCR1-HOS cells were not stimulated further by hMIP-1α or RANTES after stimulation by the recombinant Lkn-1 while further stimulation of CCR1-HOS cells by the recombinant Lkn-1 was not affected after

stimulation by hMIP-1α or RANTES.

Figure 10(A) revealed that the recombinant Lkn-1 is a stronger agonist against CCR1 than hMIP-1α or RANTES, which was clearly confirmed from calcium responses depending on concentrations of the recombinant Lkn-1 and hMIP-1α as shown in Figure 10(C).

Moreover, as shown in Figure 10(B), CCR3-HOS cells were desensitized almost completely during further stimulation by eotaxin or the recombinant Lkn-1 after the cells were stimulated by eotaxin; CCR3-HOS cells were desensitized almost completely during further stimulation by RANTES after the cells were stimulated by the recombinant Lkn-1; and, further stimulation of CCR3-HOS cells by eotaxin was, however, not affected after the cells were stimulated by the recombinant Lkn-1 or RANTES. Also, calcium responses depending on concentrations of eotaxin, the recombinant Lkn-1 and RANTES showed that the recombinant Lkn-1 is a stronger agonist against CCR3 than RANTES although it is not a stronger one than eotaxin(see: Figure 10(D)).

As clearly illustrated and demonstrated as aboves, the present invention provides a cDNA coding for a novel Lkn-1 protein which belongs to C6 β-chemokines, and an amino acid sequence deduced therefrom. Open reading frame of the Lkn-1 cDNA encodes 113 amino acids containing a signal peptide of 21 amino acids, and molecular weight of mature protein consisting of 92 amino acids among them is supposed to be 10,162 dalton. A recombinant Lkn-1 which was expressed in E. coli or insect cell employing the said Lkn-1 cDNA and purified, inhibited colony formation and cell proliferation significantly, attracted neutrophils, monocytes and lymphocytes to cause chemotaxis, and bound to CCR1 and CCR3 receptors. Accordingly, it was determined that the recombinant Lkn-1 protein can be used as a potential drug

for antibody production, the treatment during HIV-1 infection, the protection of bone marrow stem cells during chemotherapy or radiotherapy and the inhibition of leukemia, etc.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(C) CITY: Yongin, Kyonggi-Do
(D) STATE: not applicable
(E) COUNTRY: Korea
(F) POSTAL CODE (ZIP): 449-900
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(H) TELEFAX: 02-582-6331

(ii) TITLE OF INVENTION: A cDNA ENCODING C6 β-CHEMOKINE LEUKOTACTIN-1 (Lkn-1) ISOLATED FROM HUMAN

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 202 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: genomic DNA library from human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCCATCAG CAGAGAAAGG AAAAAACAGG CTGTGTTGAC TGGGAAATCT 50
GAGGAGCAGG GAGGATGGGG CCCCCTGTCT CCATCTGCCC ACACCTCAGT 100
TTGTAATCTT TCTCTCCCTT GTTCCCCAGA TTCCTCACCA AGAAGGGGCG 150

GCAAGTCTGT GCCAAACCCA GTGGTCCGGG AGTTCAGGAT TGCATGAAAA 200
AG 202

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Phe Leu Thr Lys Lys Gly Arg Gln Val Cys Ala Lys Pro Ser
1 5 10

Gly Pro Gly Val Gln Asp Cys Met Lys Lys
15 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Murine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Phe Ile Ser Lys Arg Gly Phe Gln Val Cys Ala Asn Pro Ser
1 5 10

Asp Arg Arg Val Gln Arg Cys Ile Glu Arg
15 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 582 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: DNA library from human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CGGAGCCAGG AAGCAGTGAG CCCAGGAGTC CTCGGCCAGC CCTGCCTGCC  50
CACCAGGAGG ATGAAGGTCT CCGTGGCTGC CCTCTCCTGC CTCATGCTTA 100
TTGCTGTCCT TGGATCCCAG GCCCAGTTCA CAAATGATGC AGAGACAGAG 150
TTAATGATGT CAAAGCTTCC ACTGGAAAAT CCAGTAGTTC TGAACAGCTT 200
TCACTTTGCT GCTGACTGCT GCACCTCCTA CATCTCACAA AGCATCCCGT 250
GTTCACTCAT GAAAAGTTAT TTTGAAACGA GCAGCGAGTG CTCCAAGCCA 300
GGTGTCATAT TCCTCACCAA GAAGGGGCGG CAAGTCTGTG CCAAACCCAG 350
TGGTCCGGGA GTTCAGGATT GCATGAAAAA GCTGAAGCCC TACTCAATAT 400
AATAATAAAC AGACAAAAGA GGCCAGCCAC CCACCTCCAA CACCTCCTGT 450
GAGTTTCTTG GTCTGAAATA CTTAAAAAAT ATATATATTG TTGTGTCTGG 500
TAATGAAAGT AATGCATCTA ATAAAGGTAT TCAATTTTTT AACTTTGCTT 550
GAGTTTTAAG AGGAAATAAA CTAATATAAA AC                     582
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Leu Ile
1               5                  10

Ala Val Leu Gly Ser Gln Ala Gln Phe Thr Asn Asp Ala Glu
15                  20                  25

Thr Glu Leu Met Met Ser Lys Leu Pro Leu Glu Asn Pro Val
    30                  35                  40

Val Leu Asn Ser Phe His Phe Ala Ala Asp Cys Cys Thr Ser
        45                  50                  55

Tyr Ile Ser Gln Ser Ile Pro Cys Ser Leu Met Lys Ser Tyr
            60                  65                  70
```

Phe Glu Thr Ser Ser Glu Cys Ser Lys Pro Gly Val Ile Phe
75 80

Leu Thr Lys Lys Gly Arg Gln Val Cys Ala Lys Pro Ser Gly
85 90 95

Pro Gly Val Gln Asp Cys Met Lys Lys Leu Lys Pro Tyr Ser
100 105 110

Ile

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 342 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human
(vii) IMMEDIATE SOURCE:
(A) LIBRARY: DNA library from human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGAAGGTCT CCGTGGCTGC CCTCTCCTGC CTCATGCTTA TTGCTGTCCT 50
TGGATCCCAG GCCCAGTTCA CAAATGATGC AGAGACAGAG TTAATGATGT 100
CAAAGCTTCC ACTGGAAAAT CCAGTAGTTC TGAACAGCTT TCACTTTGCT 150
GCTGACTGCT GCACCTCCTA CATCTCACAA AGCATCCCGT GTTCACTCAT 200
GAAAAGTTAT TTTGAAACGA GCAGCGAGTG CTCCAAGCCA GGTGTCATAT 250
TCCTCACCAA GAAGGGGCGG CAAGTCTGTG CCAAACCCAG TGGTCCGGGA 300
GTTCAGGATT GCATGAAAAA GCTGAAGCCC TACTCAATAT AA 342

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gln Phe Thr Asn Asp Ala Glu Thr Glu Leu Met Met Ser Lys
1 5 10

Leu Pro Leu Glu Asn Pro Val Val Leu Asn Ser Phe His Phe
15 20 25

Ala Ala Asp Cys Cys Thr Ser Tyr Ile Ser Gln Ser Ile Pro
30 35 40

Cys Ser Leu Met Lys Ser Tyr Phe Glu Thr Ser Ser Glu Cys
45 50 55

Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Lys Gly Arg Gln
60 65 70

Val Cys Ala Lys Pro Ser Gly Pro Gly Val Gln Asp Cys Met
75 80

Lys Lys Leu Lys Pro Tyr Ser Ile
85 90

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Murine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Leu Ile Gln Ile Met Glu Lys Glu Asp Arg Arg Tyr Asn
1 5 10

Pro Pro Ile Ile His Gln Gly Phe Gln Asp Thr Ser Ser Asp
15 20 25

Cys Cys Phe Ser Tyr Ala Thr Gln Ile Pro Cys Lys Arg Phe
30 35 40

Ile Tyr Tyr Phe Pro Thr Ser Gly Gly Cys Ile Lys Pro Gly
45 50 55

Ile Ile Phe Ile Ser Arg Arg Gly Thr Gln Val Cys Ala Asp
60 65 70

Pro Ser Asp Arg Arg Val Gln Arg Cys Leu Ser Thr Leu Lys
75 80

Gln Gly Pro Arg Ser Gly Asn Lys Val Ile Ala
85 90 95

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Murine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gln Ile Thr His Ala Thr Glu Thr Lys Glu Val Gln Ser Ser
1 5 10

Leu Lys Ala Gln Gln Gly Leu Glu Ile Glu Met Phe His Met
15 20 25

Gly Phe Gln Asp Ser Ser Asp Cys Cys Leu Ser Tyr Asn Ser
30 35 40

Arg Ile Gln Cys Ser Arg Phe Ile Gly Tyr Phe Pro Thr Ser
45 50 55

Gly Gly Cys Thr Arg Pro Gly Ile Ile Phe Ile Ser Lys Arg
60 65 70

Gly Phe Gln Val Cys Ala Asn Pro Ser Asp Arg Arg Val Gln
75 80

Arg Cys Ile Glu Arg Leu Glu Gln Asn Ser Gln Pro Arg Thr
85 90 95

Tyr Tyr Lys
100

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Ser Leu Ala Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser
1 5 10

Tyr Thr Ser Arg Gln Ile Pro Gln Asn Phe Ile Ala Asp Tyr
15 20 25

Phe Glu Thr Ser Ser Gln Cys Ser Lys Pro Gly Val Ile Phe
30 35 40

Leu Thr Lys Arg Ser Arg Gln Val Cys Ala Asp Pro Ser Glu
45 50 55

Glu Trp Val Gln Lys Tyr Val Ser Asp Leu Glu
60 65

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Murine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Pro Tyr Gly Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser
1 5 10

Tyr Ser Arg Lys Ile Pro Arg Gln Phe Ile Val Glu Val Phe
15 20 25

Glu Thr Ser Ser Leu Cys Ser Gln Pro Gly Val Ile Phe Leu
30 35 40

Thr Lys Arg Asn Arg Gln Ile Cys Ala Asp Ser Lys Glu Thr
45 50 55

Trp Val Gln Glu Tyr Ile Thr Asp Leu Glu
60 65

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Pro Met Gly Ser Asp Pro Pro Thr Ala Cys Cys Phe Ser
1 5 10

Tyr Thr Ala Arg Lys Leu Pro Arg Asn Phe Val Val Asp Tyr
15 20 25

Tyr Glu Thr Ser Ser Leu Cys Ser Gln Pro Ala Val Val Phe
30 35 40

Gln Thr Lys Arg Ser Lys Gln Val Cys Ala Asp Pro Ser Glu
45 50 55

Ser Trp Val Gln Glu Val Tyr Tyr Asp Leu Glu
60 65

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTCCTCACCA AGAAGGGG 18

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:

(B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTTTTCATG CAATCCTG 18

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGAATTCCAT ATGCAGTTCA CAAATGATGC AGAG 34

(2) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCCGCTCGA GTTGAGTAGG GCTTCAGC 28

WHAT IS CLAIMED IS:

1. A cDNA of human C6 β-chemokine Lkn-1(leukotactin-1) whose nucleotide sequence is represented as following(SEQ ID NO:6):

```
ATG AAG GTC TCC GTG GCT GCC CTC TCC TGC CTC ATG CTT  39
ATT GCT GTC CTT GGA TCC CAG GCC CAG TTC ACA AAT GAT  78
GCA GAG ACA GAG TTA ATG ATG TCA AAG CTT CCA CTG GAA 117
AAT CCA GTA GTT CTG AAC AGC TTT CAC TTT GCT GCT GAC 156
TGC TGC ACC TCC TAC ATC TCA CAA AGC ATC CCG TGT TCA 195
CTC ATG AAA AGT TAT TTT GAA ACG AGC AGC GAG TGC TCC 234
AAG CCA GGT GTC ATA TTC CTC ACC AAG AAG GGG CGG CAA 273
GTC TGT GCC AAA CCC AGT GGT CCG GGA GTT CAG GAT TGC 312
ATG AAA AAG CTG AAG CCC TAC TCA ATA TAA             342
```

2. Human C6 β-chemokine Lkn-1(leukotactin-1) whose amino acid sequence is represented as following(SEQ ID NO:5):

```
Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Leu Ile
1               5                  10

Ala Val Leu Gly Ser Gln Ala Gln Phe Thr Asn Asp Ala Glu
15                  20                  25

Thr Glu Leu Met Met Ser Lys Leu Pro Leu Glu Asn Pro Val
    30                  35                  40

Val Leu Asn Ser Phe His Phe Ala Ala Asp Cys Cys Thr Ser
        45                  50                  55

Tyr Ile Ser Gln Ser Ile Pro Cys Ser Leu Met Lys Ser Tyr
            60                  65                  70

Phe Glu Thr Ser Ser Glu Cys Ser Lys Pro Gly Val Ile Phe
                75                  80

Leu Thr Lys Lys Gly Arg Gln Val Cys Ala Lys Pro Ser Gly
85                  90                  95

Pro Gly Val Gln Asp Cys Met Lys Lys Leu Lys Pro Tyr Ser
    100                 105                 110
```

Ile

3. A cDNA of human C6 β-chemokine Lkn-1 (leukotactin-1) from which the 1st to 63th nucleotide sequences are deleted.

4. An expression vector which comprises the cDNA of claim 1.

5. An expression vector PVL 1392-Lkn-1 which comprises the cDNA of claim 1.

6. A process for preparing a recombinant leukotactin-1 which comprises the steps of transforming a host cell with the expression vector of claim 4, culturing the transformant, and obtaining the recombinant Lkn-1 therefrom.

7. The process of claim 6 wherein the host cell is E. coli or insect cell.

8. A recombinant leukotactin-1 prepared by a process which comprises the steps of transforming a host cell with the expression vector of claim 4, culturing the transformant, and obtaining the recombinant Lkn-1 from the culture.

9. The recombinant leukotactin-1 of claim 8 which inhibits colony formation.

10. The recombinant leukotactin-1 of claim 8 which attracts human peripheral blood neutrophils, monocytes and lymphocytes strongly to cause chemotaxis.

11. The recombinant leukotactin-1 of claim 8 which binds to CCR1 and CCR3 receptors.

12. The recombinant leukotactin-1 of claim 11 which shares receptors with RANTES(regulated activated normal T cell expressed sequence) and hMIP-1α(human macrophage inflammatory protein-1α) and does not share receptors with IL-8(interleukin-8).

13. An expression vector which comprises the cDNA of claim 3.

14. An expression vector pET21a-Lkn-1 which comprises the cDNA of claim 3.

15. Escherichia coli (XL-1 Blue) hMRP-2 transformed with the expression vector of claim 14(ATCC 98166).

16. A process for preparing a recombinant leukotactin-1 which comprises the steps of transforming a host cell with the expression vector of claim 13, culturing the transformant, and obtaining the recombinant Lkn-1 therefrom.

17. The process of claim 16 wherein the host cell is E. coli or insect cell.

18. A recombinant leukotactin-1 prepared by a process which comprises the steps of transforming a host cell with the expression vector of claim 13, culturing the transformant, and obtaining the recombinant Lkn-1 therefrom.

19. The recombinant leukotactin-1 of claim 18 which inhibits colony formation.

20. The recombinant leukotactin-1 of claim 18 which attracts human peripheral blood neutrophils, monocytes and lymphocytes strongly to cause chemotaxis.

21. The recombinant leukotactin-1 of claim 18 which binds to CCR1 and CCR3 receptors.

22. The recombinant leukotactin-1 of claim 21 which shares receptors with RANTES(regulated activated normal T cell expressed sequence) and hMIP-1α(human macrophage inflammatory protein-1α) and does not share receptors with IL-8(interleukin-8).

23. A method for protecting myeloid cells from cytotoxic anti-cancer drugs or radiation which comprises a step of administrating human C6 β-chemokine Lkn-1(leukotactin-1) into a subject.

24. The method of claim 23, wherein the human C6 β-chemokine Lkn-1(leukotactin-1) is the recombinant leukotactin-1 of claim 8.

25. The method of claim 23, wherein the human C6 β-chemokine Lkn-1(leukotactin-1) is the recombinant leukotactin-1 of claim 18.

5' - CTGCCATCAGCAGAGAAAGGAAAAAACAGGCTGTGTTGACTGGGAAATCTGAGGAGCAGG
GAGGATGGGGCCCCCTGTCTCCATCTGCCCACACCTCAGTTTGTAATCTTTCTCTCCCTTGT
TCCCCAGA TTC CTC ACC AAG AAG GGG CGG CAA GTC TGT GCC AAA CCC
intron F L T K K G R Q V C A K P
AGT GGT CCG GGA GTT CAG GAT TGC ATG AAA AAG - 3'
S G P G V Q D C M K K

Fig. 1(A)

Murine MRP-2	(87)	F	I	S	K	R	G	F	Q	V	C	A	N	P	S	D	R	R		
Lkn-1		F	L	T	K	K	G	R	Q	V	C	A	K	P	S	G	P	G		

Murine MRP-2	V	Q	R	C	I	E	R	(110)
Lkn-1	V	Q	D	C	M	K	K	

Fig. 1(B)

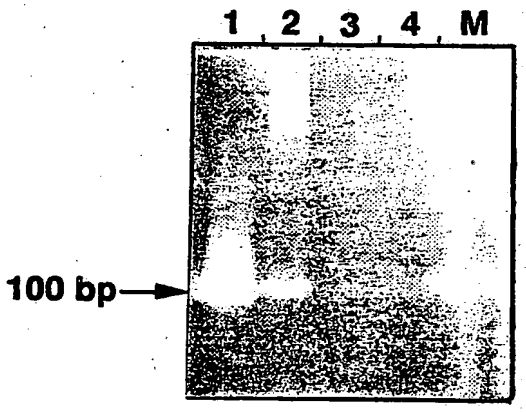


Fig. 2

```
-60  CGGAGCCAGG AAGCAGTGAG CCCAGGAGTC CTCGGCCAGC CCTGCCTGCC CACCAGGAGG
 1 ATG AAG GTC TCC GTG GCT GCC CTC TCC TGC CTC ATG CTT ATT GCT GTC CTT
 1  M   K   V   S   V   A   A   L   S   C   L   M   L   I   A   V   L
52 GGA TCC CAG GCC CAG TTC ACA AAT GAT GCA GAG ACA GAG TTA ATG ATG TCA
18  G   S   Q   A   Q   F   T   N   D   A   E   T   E   L   M   M   S
103 AAG CTT CCA CTG GAA AAT CCA GTA GTT CTG AAC AGC TTT CAC TTT GCT GCT
35  K   L   P   L   E   N   P   V   V   L   N   S   F   H   F   A   A
154 GAC TGC TGC ACC TCC TAC ATC TCA CAA AGC ATC CCG TGT TCA CTC ATG AAA
52  D   C   C   T   S   Y   I   S   Q   S   I   P   C   S   L   M   K

205 AGT TAT TTT GAA ACG AGC AGC GAG TGC TCC AAG CCA GGT GTC ATA TTC CTC
69  S   Y   F   E   T   S   S   E   C   S   K   P   G   V   I   F   L

256 ACC AAG AAG GGG CGG CAA GTC TGT GCC AAA CCC AGT GGT CCG GGA GTT CAG
86  T   K   K   G   R   Q   V   C   A   K   P   S   G   P   G   V   Q

307 GAT TGC ATG AAA AAG CTG AAG CCC TAC TCA ATA TAA TAA TAAAC AGACAAAAGA
103 D   C   M   K   K   L   K   P   Y   S   I  ***

361 GGCCAGCCAC CCACCTCCAA CACCTCCTGT GAGTTTCTTG GTCTGAAATA CTTAAAAAAT
421 ATATATATTG TTGTGTCTGG TAATGAAAGT AATGCATCTA ATAAAGGTAT TCAATTTTTT
481 AACTTTGCTT GAGTTTTAAG AGGAAATAAA CTAATATAAA AC
```

Fig. 3

```
Lkn-1      QFTNDAETEL M-----MSKLP L----------  ---EHPVVLL- NSFHF-AAEC CFSYISQSIP   42
mMRP-1     G-----LIQE ME---KEDNR Y----------  ---HPPTTH- QGFQDTSSIC CFSYATQ-IP   37
mMRP-2     QITHATETKE VQSSLKAQQG L----------  ---ETEAEH- MGFQD-SSIC CLSYNSR-IQ   45
hMIP-1α    A---------- ---------- ----------  ---------SL A-A-DTPTAC CFSYTSRQIP   21
mMIP-1α    A---------- ---------- ----------  --------PY G-A-DTPTAC CFSYSNK-IP   20
hMIP-1β    A---------- ---------- ----------  --------PH G-S-DQPTAC CFSYTARKLP   21

Lkn-1      ĊSLAHKSYFET SSICSKPGVI FLTKKGRQVC AKPSGPGVQD ĊMKKLK---- ---PYSI     92
mMRP-1     CKHFIYYFPT SGCCIKPGII FTSRRGTQVC ADPSDRRVQR CLSTLKQGPR SGNKVIA     95
mMRP-2     CSRFIGYFPT SGCCIRPGII FTSKRGFQVC ANPSDRRVQR CIERLEQNSQ P-RTYYK    101
hMIP-1α    QNFIADYFET SSCCSKPGVI FLTKRSRQVC ADPSEEWVQK YVSDLE----          70
mMIP-1α    RQFIVEVFET SSICSQPGVI FLTKRNRQIC ADSKETWVQE YITDLE----          69
hMIP-1β    RNFVVDYYET SSICSQPAVV FQTKRSKQVC ADPSESWVQE VVYDLE----          69
```

Fig. 4

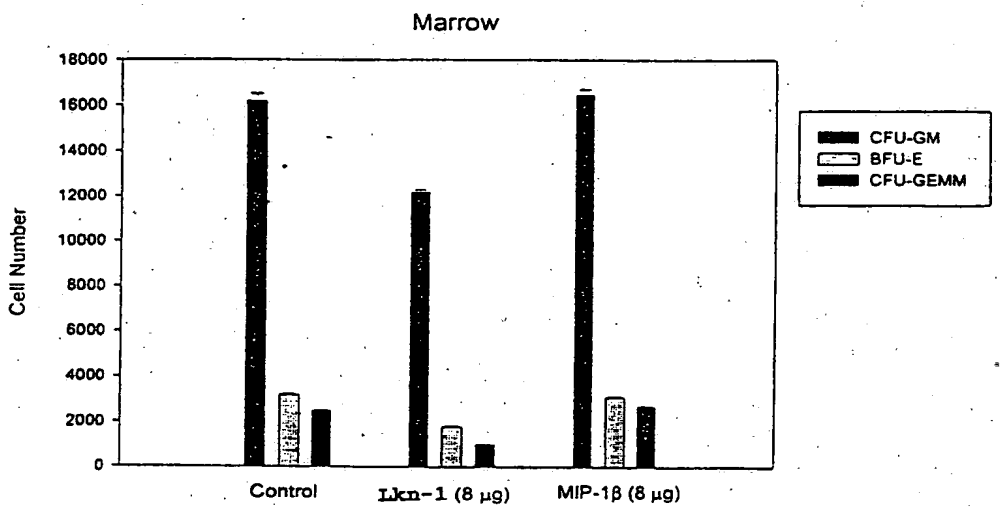

Marrow
Cell Number
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Control
Lkn-1 (8 μg)
MIP-1β (8 μg)
CFU-GM
BFU-E
CFU-GEMM

Fig.5(A)

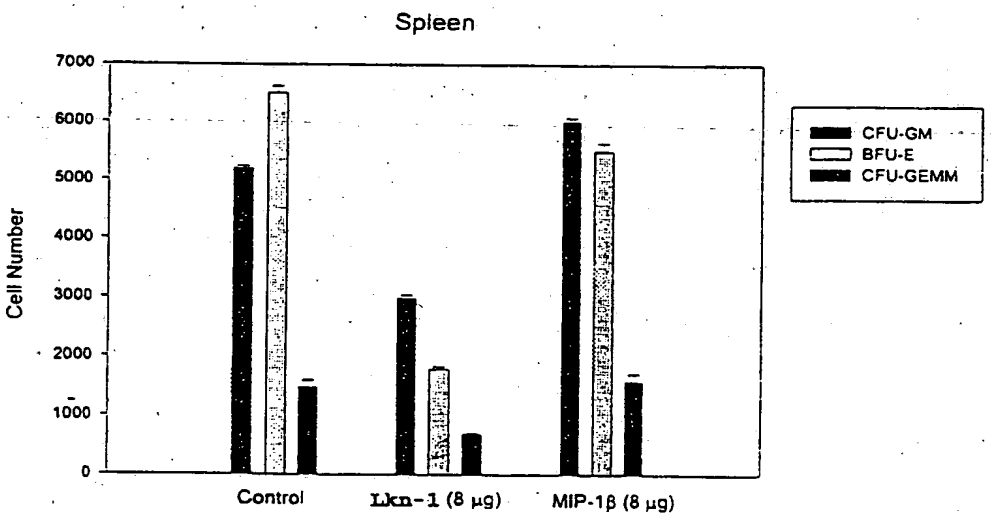

Spleen
Cell Number
7000
6000
5000
4000
3000
2000
1000
0
Control
Lkn-1 (8 μg)
MIP-1β (8 μg)
CFU-GM
BFU-E
CFU-GEMM

Fig.5(B)

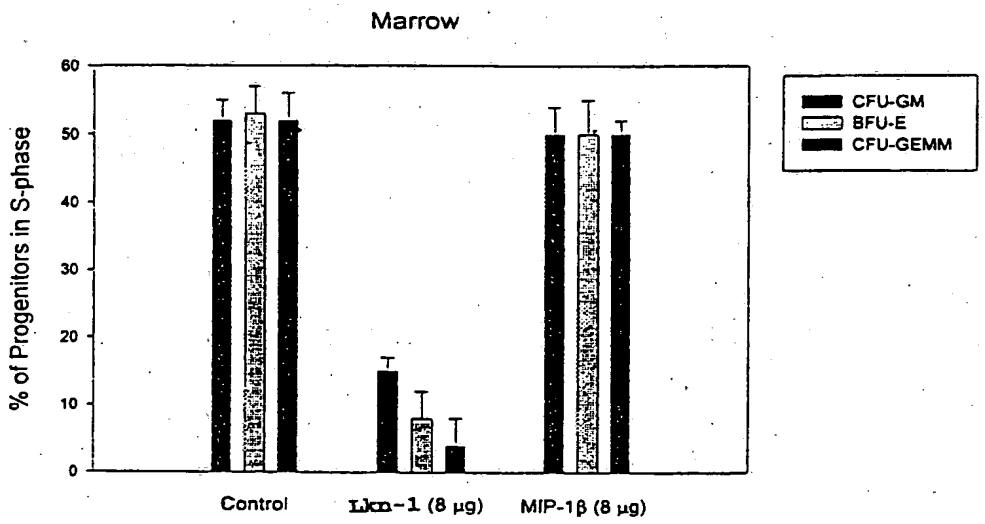

Marrow
% of Progenitors in S-phase
60
50
40
30
20
10
0
Control
Lkn-1 (8 μg)
MIP-1β (8 μg)
CFU-GM
BFU-E
CFU-GEMM

Fig.5(C)

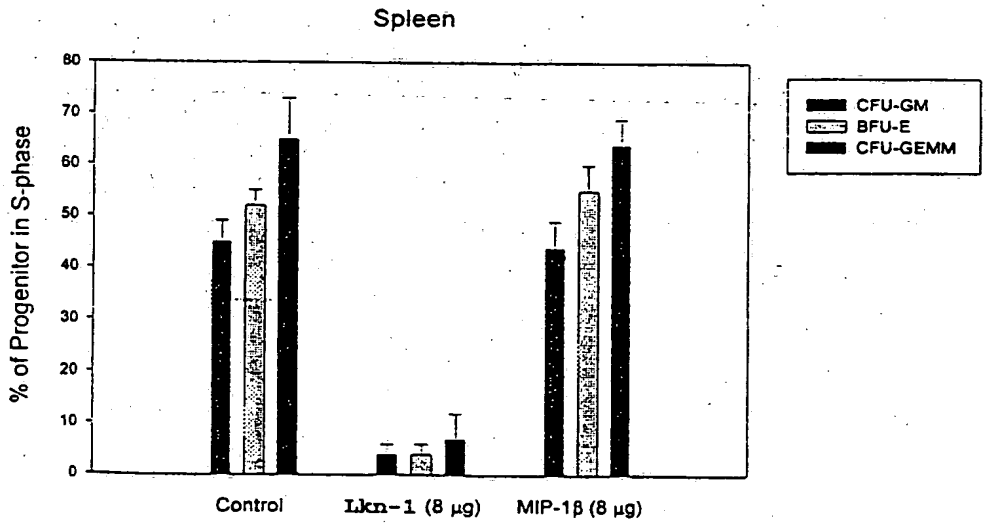

Spleen
% of Progenitor in S-phase
80
70
60
50
40
30
20
10
0
Control
Lkn-1 (8 μg)
MIP-1β (8 μg)
CFU-GM
BFU-E
CFU-GEMM

Fig.5(D)

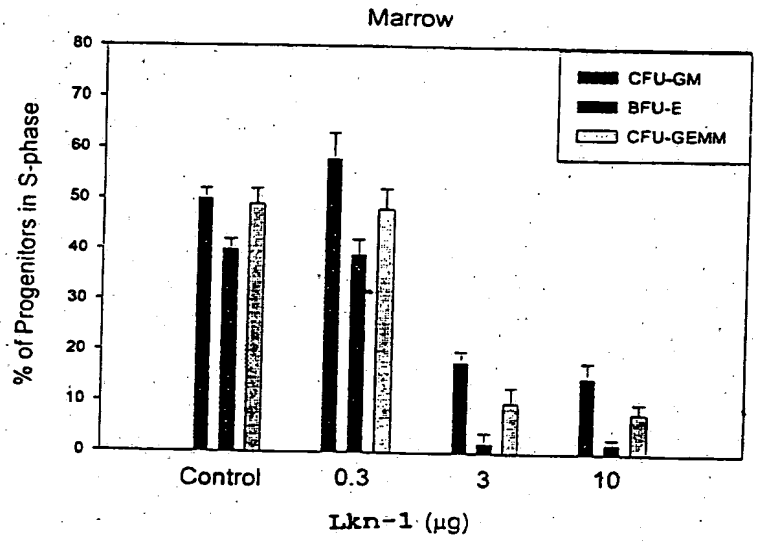

Marrow
% of Progenitors in S-phase
80
70
60
50
40
30
20
10
0
CFU-GM
BFU-E
CFU-GEMM
Control
0.3
3
10
Lkn-1 (μg)

Fig.6(A)

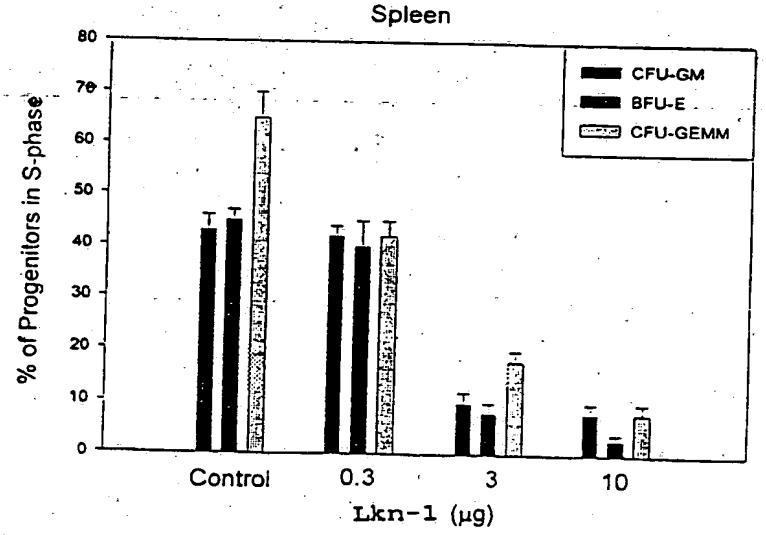

Spleen
% of Progenitors in S-phase
80
70
60
50
40
30
20
10
0
CFU-GM
BFU-E
CFU-GEMM
Control
0.3
3
10
Lkn-1 (μg)

Fig.6(B)

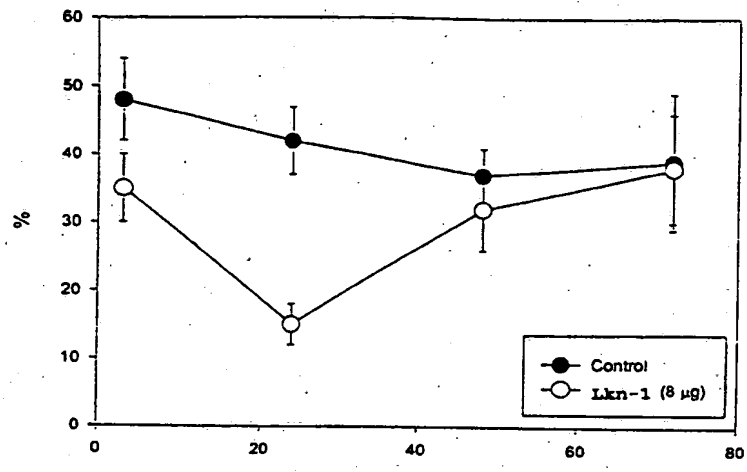


Fig.7(A)

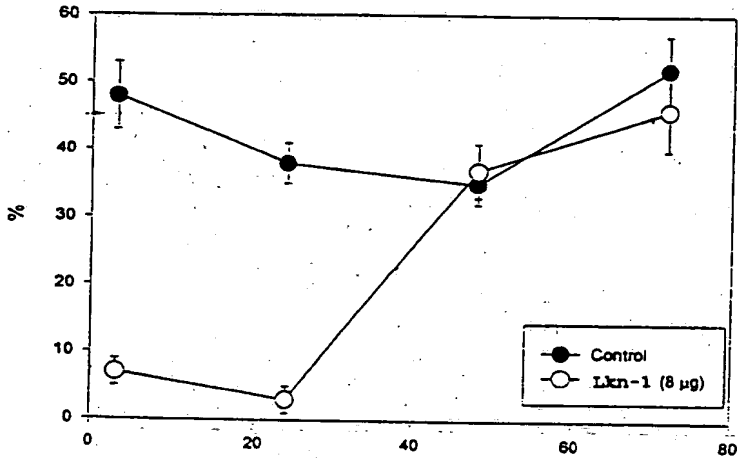


Fig.7(B)

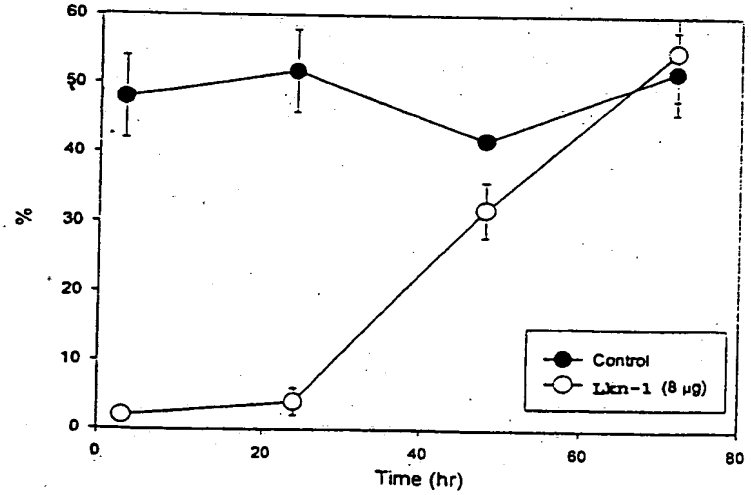


Fig.7(C)

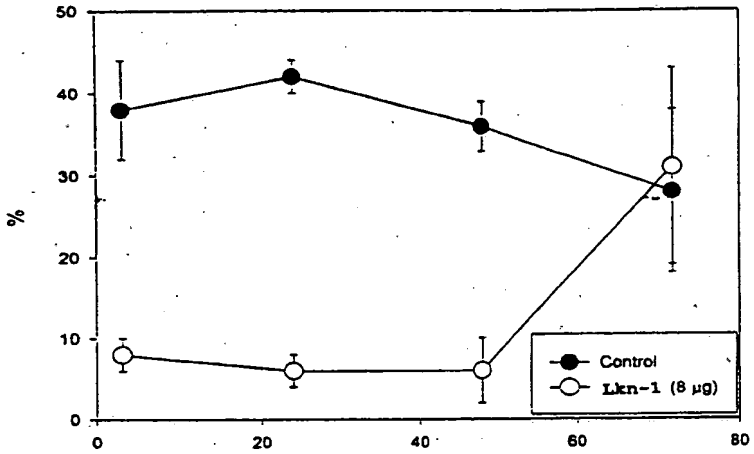


Fig.7(D)

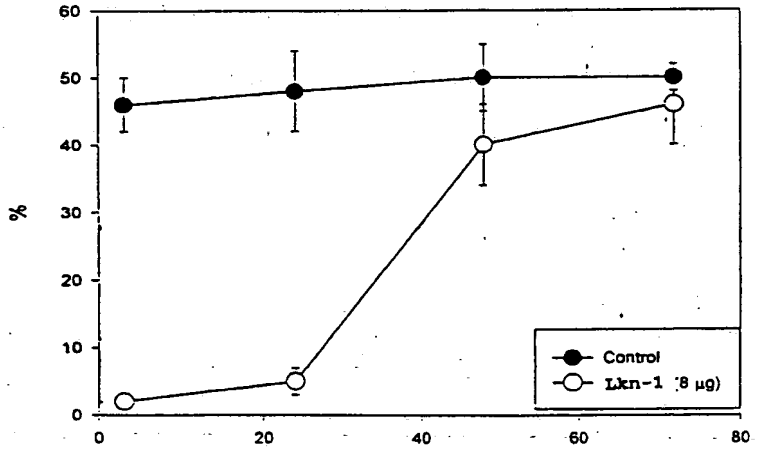


Fig.7(E)

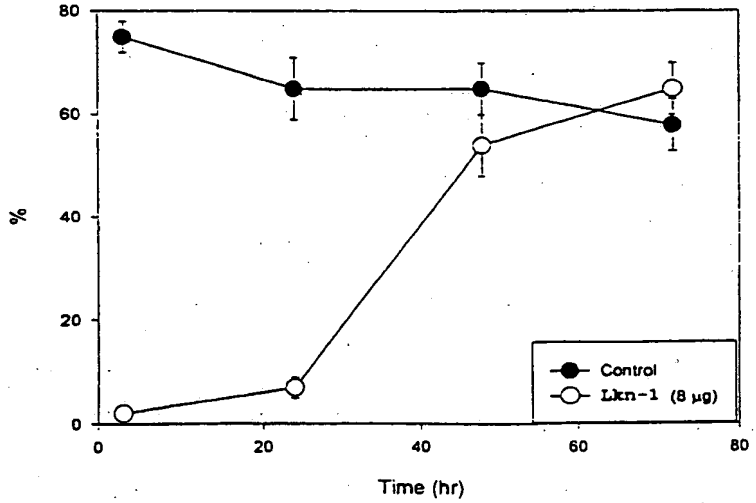


Fig.7(F)

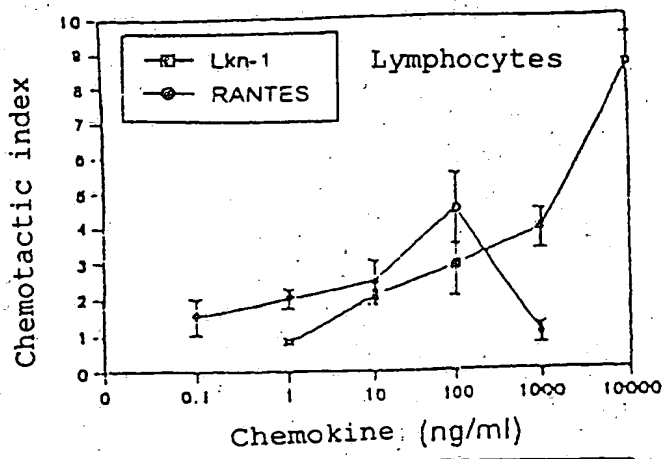


Fig.8(A)

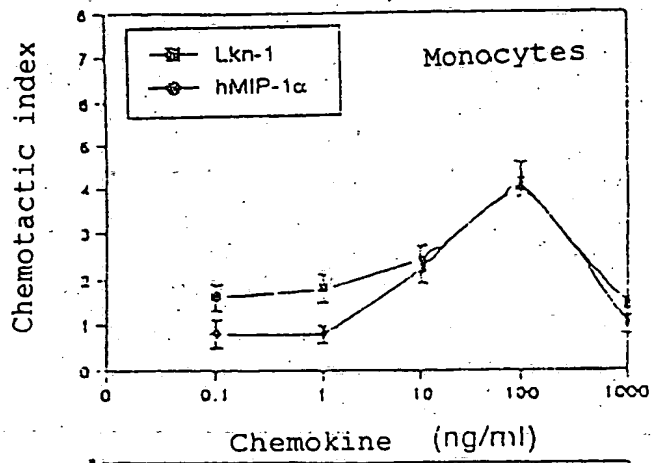


Fig.8(B)

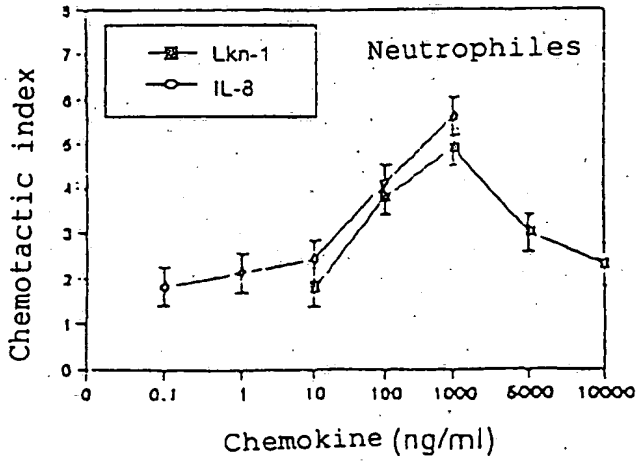


Fig.8(C)

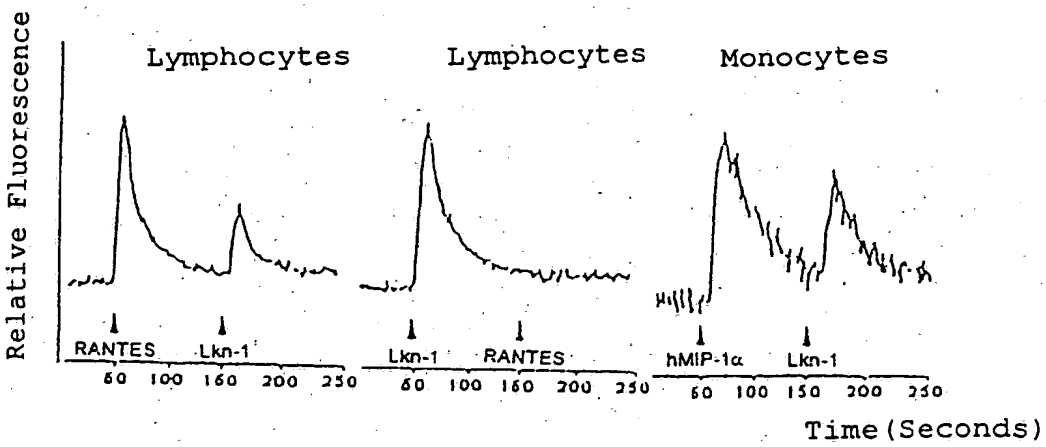


Fig.9(A) Fig.9(B) Fig.9(C)

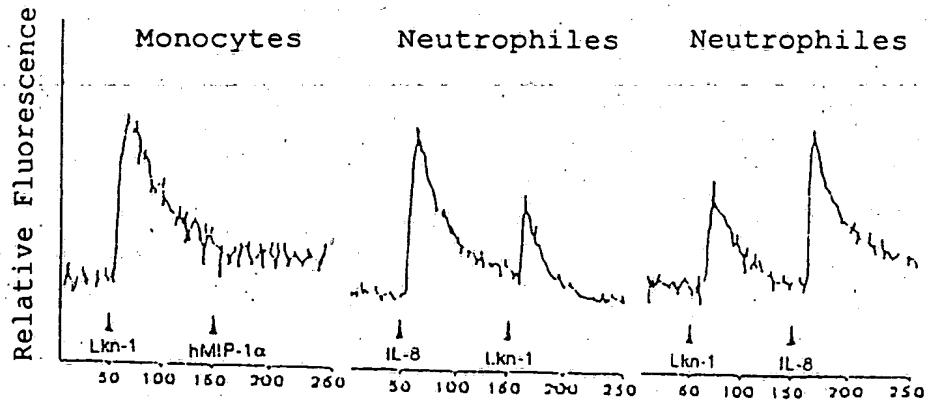


Fig.9(D) Fig.9(E) Fig.9(F)

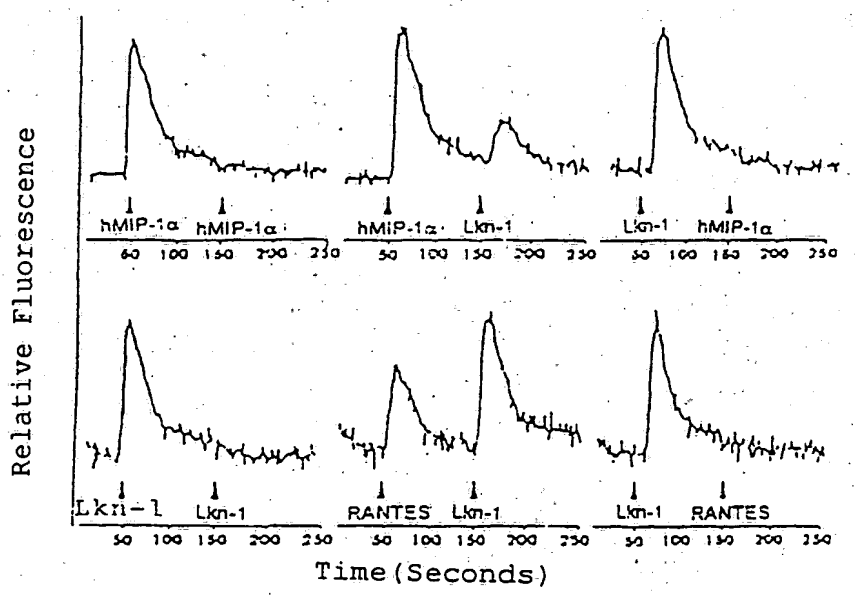


Fig.10(A)

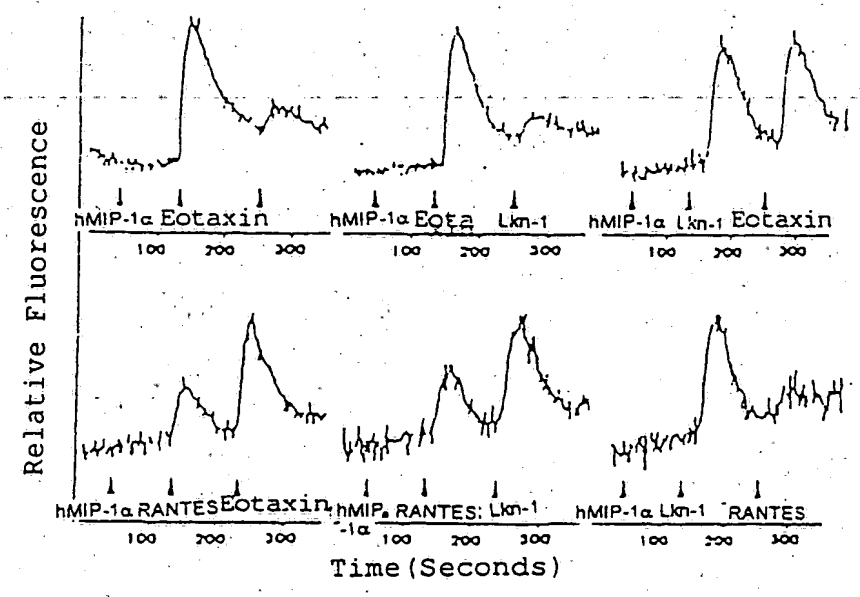


Fig.10(B)

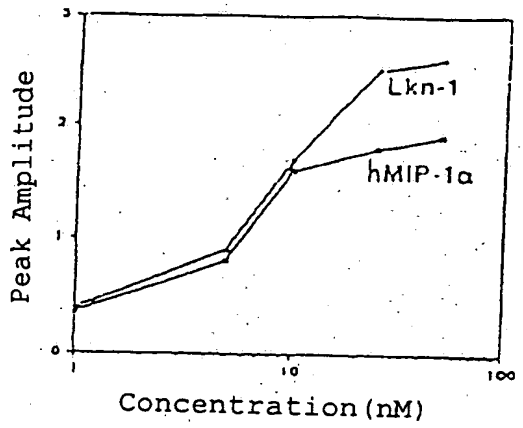


Fig.10(C)

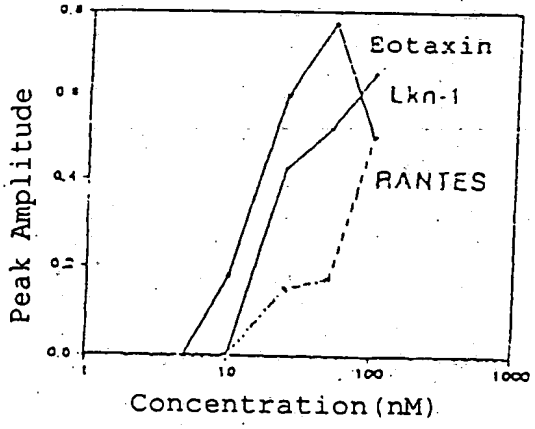


Fig.10(D)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00381

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C12 N 15/19, C 07 K 14/52, C 12 N 5/10, A 61 K 38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/19, C 07 K 14/52, C 12 N 5/10, A 61 K 38/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97/39 126 A1 (INCYTE PHARMACEUTICALS, INC.) 23 October 1997 (23.10.97), abstract; claims.	1-25
A	WO 97/12 041 A1 (SMITHKLINE BEECHAM CORPORATION) 03 April 1997 (03.04.97), abstract; claims.	1-25
A	WO 96/39 522 A1 (HUMAN GENOME SCIENCES INC.) 12 December 1996 (12.12.96), abstract; claims.	1-25
A	WO 96/39 521 A1 (HUMAN GENOME SCIENCES, INC.) 12 December 1996 (12.12.96), abstract; claims.	1-25
A	WO 96/39 520 A1 (HUMAN GENOME SCIENCES, INC.) 12 December 1996 (12.12.96), abstract; claims.	1-25

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 1999 (30.03.99)	13 April 1999 (13.4.99)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/KR 98/00381

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 23-25 concern a method of treatment of the human or animal body by therapy (see Rule 39.1iv PCT) the search was carried out and based on the alleged effects.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 98/00381

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9739126	23-10-97	AU A1 28023/97 US A 5840544	07-11-97 24-11-98
WO A1 9712041	03-04-97	AU A1 73790/96 CN A 1198186 CZ A3 9800921 EP A1 859842 EP A4 859842 NO A0 981387 NO A 981387 PL A1 326080	17-04-97 04-11-98 15-07-98 26-08-98 24-02-99 26-03-98 29-05-98 17-08-98
WO A1 9639522	12-12-96	AU A1 61628/96 CA AA 2222280 CN A 1190991 EP A1 833914	24-12-96 12-12-96 19-08-98 08-04-98
WO A1 9639521	12-12-96	CA AA 2217216 AU A1 28208/95 EP A1 832233 EP A4 832233	12-12-96 24-12-96 01-04-98 01-07-98
WO A1 9639520	12-12-96	AU A1 26987/95	24-12-96

INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/US 98/26236

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9735027 A	25-09-1997	AU 5425496 A	10-10-1997
		EP 0904398 A	31-03-1999

cells such as granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelo-suppression; in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentary to platelet transfusions; and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility in various stem cell disorders (such as those usually treated with transplantation, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either in-vivo or ex-vivo (i.e., in conjunction with bone marrow transplantation or with peripheral progenitor cell transplantation (homologous or heterologous)) as normal cells or genetically manipulated for gene therapy.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for proliferation and differentiation of various hematopoietic lines are cited above.

Assays for embryonic stem cell differentiation (which will identify, among others, proteins that influence embryonic differentiation hematopoiesis) include, without limitation, those described in: Johansson et al. Cellular Biology 15:141-151, 1995; Keller et al., Molecular and Cellular Biology 13:473-486, 1993; McClanahan et al., Blood 81:2903-2915, 1993.

Assays for stem cell survival and differentiation (which will identify, among others, proteins that regulate lympho-hematopoiesis) include, without limitation, those described in: Methylcellulose colony forming assays, Freshney, M. G. In Culture of Hematopoietic Cells. R. I. Freshney, et al. eds. Vol pp. 265-268, Wiley-Liss, Inc., New York, N.Y. 1994; Hirayama et al., Proc. Natl. Acad. Sci. USA 89:5907-5911, 1992; Primitive hematopoietic colony forming cells with high proliferative potential, McNiece, I.

K. and Briddell, R. A. In Culture of Hematopoietic Cells. R. I. Freshney, et al. eds. Vol pp. 23-39, Wiley-Liss, Inc., New York, N.Y. 1994; Neben et al., Experimental Hematology 22:353-359, 1994; Cobblestone area forming cell assay, Ploemacher, R. E. In Culture of Hematopoietic Cells. R. I. Freshney, et al. eds. Vol pp. 1-21, Wiley-Liss, Inc., New York, N.Y. 1994; Long term bone marrow cultures in the presence of stromal cells, Spooncer, E., Dexter, M. and Allen, T. In Culture of Hematopoietic Cells. R. I. Freshney, et al. eds. Vol pp. 163-179, Wiley-Liss, Inc., New York, N.Y. 1994; Long term culture initiating cell assay, Sutherland, H. J. In Culture of Hematopoietic Cells. R. I. Freshney, et al. eds. Vol pp. 139-162, Wiley-Liss, Inc., New York, N.Y. 1994.

5.5.6. TISSUE GROWTH ACTIVITY

A protein of the present invention also may have utility in compositions used for bone, cartilage, tendon, ligament and/or nerve tissue growth or regeneration, as well as for wound healing and tissue repair and replacement, and in the treatment of burns, incisions and ulcers.

A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage damage or defects in humans and other animals. Such a preparation employing a protein of the invention may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery.

A protein of this invention may also be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A protein of the invention may also be useful in the treatment of osteoporosis or osteoarthritis, such as through stimulation of bone and/or cartilage repair or by blocking inflammation or processes of tissue destruction (collagenase

activity, osteoclast activity, etc.) mediated by inflammatory processes.

Another category of tissue regeneration activity that may be attributable to the protein of the present invention is tendon/ligament formation. A protein of the present invention, which induces tendon/ligament-like tissue or other tissue formation in circumstances where such tissue is not normally formed, has application in the healing of tendon or ligament tears, deformities and other tendon or ligament defects in humans and other animals. Such a preparation employing a tendon/ligament-like tissue inducing protein may have prophylactic use in preventing damage to tendon or ligament tissue, as well as use in the improved fixation of tendon or ligament to bone or other tissues, and in repairing defects to tendon or ligament tissue. De novo tendon/ligament-like tissue formation induced by a composition of the present invention contributes to the repair of congenital, trauma induced, or other tendon or ligament defects of other origin, and is also useful in cosmetic plastic surgery for attachment or repair of tendons or ligaments. The compositions of the present invention may provide environment to attract tendon- or ligament-forming cells, stimulate growth of tendon- or ligament-forming cells, induce differentiation of progenitors of tendon- or ligament-forming cells, or induce growth of tendon/ligament cells or progenitors ex vivo for return in vivo to effect tissue repair. The compositions of the invention may also be useful in the treatment of tendinitis, carpal tunnel syndrome and other tendon or ligament defects. The compositions may also include an appropriate matrix and/or sequestering agent as a carrier as is well known in the art.

The protein of the present invention may also be useful for proliferation of neural cells and for regeneration of nerve and brain tissue, i.e. for the treatment of central and peripheral nervous system diseases and neuropathies, as well as mechanical and traumatic disorders, which involve degeneration, death or trauma to neural cells or nerve tissue. More specifically, a protein may be used in the treatment of diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome. Further conditions which may be treated in accordance with the present invention include

mechanical and traumatic disorders, such as spinal cord disorders, head trauma and cerebrovascular diseases such as stroke. Peripheral neuropathies resulting from chemotherapy or other medical therapies may also be treatable using a protein of the invention.

Proteins of the invention may also be useful to promote better or faster closure of non-healing wounds, including without limitation pressure ulcers, ulcers associated with vascular insufficiency, surgical and traumatic wounds, and the like.

It is expected that a protein of the present invention may also exhibit activity for generation or regeneration of other tissues, such as organs (including, for example, pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac) and vascular (including vascular endothelium) tissue, or for promoting the growth of cells comprising such tissues. Part of the desired effects may be by inhibition or modulation of fibrotic scarring to allow normal tissue to regenerate. A protein of the invention may also exhibit angiogenic activity.

A protein of the present invention may also be useful for gut protection or regeneration and treatment of lung or liver fibrosis, reperfusion injury in various tissues, and conditions resulting from systemic cytokine damage.

A protein of the present invention may also be useful for promoting or inhibiting differentiation of tissues described above from precursor tissues or cells; or for inhibiting the growth of tissues described above.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for tissue generation activity include, without limitation, those described in: International Patent Publication No. WO95/16035 (bone, cartilage, tendon); International Patent Publication No. WO95/05846 (nerve, neuronal); International Patent Publication No. WO91/07491 (skin, endothelium).

Assays for wound healing activity include, without limitation, those described in: Winter, Epidermal Wound Healing, pps. 71-112 (Maibach, H. I. and Rovee, D. T., eds.), Year Book Medical Publishers, Inc., Chicago, as modified by Eaglstein and Mertz, J.

Invest. Dermatol 71:382-84 (1978).

5.5.7. ACTIVIN/INHIBIN ACTIVITY

A protein of the present invention may also exhibit activin- or inhibin-related activities. A polynucleotide of the invention may encode a polypeptide exhibiting such characteristics. Inhibins are characterized by their ability to inhibit the release of follicle stimulating hormone (FSH), while activins and are characterized by their ability to stimulate the release of follicle stimulating hormone (FSH). Thus, a protein of the present invention, alone or in heterodimers with a member of the inhibin α-family, may be useful as a contraceptive based on the ability of inhibins to decrease fertility in female mammals and decrease spermatogenesis in male mammals. Administration of sufficient amounts of other inhibins can induce infertility in these mammals. Alternatively, the protein of the invention, as a homodimer or as a heterodimer with other protein subunits of the inhibin-β group, may be useful as a fertility inducing therapeutic, based upon the ability of activin molecules in stimulating FSH release from cells of the anterior pituitary. See, for example, U.S. Pat. No. 4,798,885. A protein of the invention may also be useful for advancement of the onset of fertility in sexually immature mammals, so as to increase the lifetime reproductive performance of domestic animals such as cows, sheep and pigs.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for activin/inhibin activity include, without limitation, those described in: Vale et al., Endocrinology 91:562-572, 1972; Ling et al., Nature 321:779-782, 1986; Vale et al., Nature 321:776-779, 1986; Mason et al., Nature 318:659-663, 1985; Forage et al., Proc. Natl. Acad. Sci. USA 83:3091-3095, 1986.

5.5.8. CHEMOTACTIC/CHEMOKINETIC ACTIVITY

A protein of the present invention may have chemotactic or chemokinetic activity (e.g., act as a chemokine) for mammalian cells, including, for example, monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells.

A polynucleotide of the invention can encode a polypeptide exhibiting such attributes. Chemotactic and chemokinetic proteins can be used to mobilize or attract a desired cell population to a desired site of action. Chemotactic or chemokinetic proteins provide particular advantages in treatment of wounds and other trauma to tissues, as well as in treatment of localized infections. For example, attraction of lymphocytes, monocytes or neutrophils to tumors or sites of infection may result in improved immune responses against the tumor or infecting agent.

A protein or peptide has chemotactic activity for a particular cell population if it can stimulate, directly or indirectly, the directed orientation or movement of such cell population. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells. Whether a particular protein has chemotactic activity for a population of cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for chemotactic activity (which will identify proteins that induce or prevent chemotaxis) consist of assays that measure the ability of a protein to induce the migration of cells across a membrane as well as the ability of a protein to induce the adhesion of one cell population to another cell population. Suitable assays for movement and adhesion include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Marguiles, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 6.12, Measurement of alpha and beta Chemokines 6.12.1-6.12.28; Taub et al. J. Clin. Invest. 95:1370-1376, 1995; Lind et al. APMIS 103:140-146, 1995; Muller et al Eur. J. Immunol. 25:1744-1748; Gruber et al. J. of Immunol. 152:5860-5867, 1994; Johnston et al. J. of Immunol. 153:1762-1768, 1994.

5.5.9. HEMOSTATIC AND THROMBOLYTIC ACTIVITY

A protein of the invention may also exhibit hemostatic or thrombolytic activity. A polynucleotide of the invention can encode a polypeptide exhibiting such attributes. Such

a protein is expected to be useful in treatment of various coagulation disorders (including hereditary disorders, such as hemophilias) or to enhance coagulation and other hemostatic events in treating wounds resulting from trauma, surgery or other causes. A protein of the invention may also be useful for dissolving or inhibiting formation of thromboses and for treatment and prevention of conditions resulting therefrom (such as, for example, infarction of cardiac and central nervous system vessels (e.g., stroke).

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assay for hemostatic and thrombolytic activity include, without limitation, those described in: Linet et al., J. Clin. Pharmacol. 26:131-140, 1986; Burdick et al., Thrombosis Res. 45:413-419, 1987; Humphrey et al., Fibrinolysis 5:71-79 (1991); Schaub, Prostaglandins 35:467-474, 1988.

5.5.10. RECEPTOR/LIGAND ACTIVITY

A protein of the present invention may also demonstrate activity as receptors, receptor ligands or inhibitors or agonists of receptor/ligand interactions. A polynucleotide of the invention can encode a polypeptide exhibiting such characteristics. Examples of such receptors and ligands include, without limitation, cytokine receptors and their ligands, receptor kinases and their ligands, receptor phosphatases and their ligands, receptors involved in cell-cell interactions and their ligands (including without limitation, cellular adhesion molecules (such as selectins, integrins and their ligands) and receptor/ligand pairs involved in antigen presentation, antigen recognition and development of cellular and humoral immune responses). Receptors and ligands are also useful for screening of potential peptide or small molecule inhibitors of the relevant receptor/ligand interaction. A protein of the present invention (including, without limitation, fragments of receptors and ligands) may themselves be useful as inhibitors of receptor/ligand interactions.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for receptor-ligand activity include without limitation those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 7.28, Measurement of Cellular Adhesion under static conditions 7.28.1-7.28.22), Takai et al., Proc. Natl. Acad. Sci. USA 84:6864-6868, 1987; Bierer et al., J. Exp. Med. 168:1145-1156, 1988; Rosenstein et al., J. Exp. Med. 169:149-160 1989; Stoltenborg et al., J. Immunol. Methods 175:59-68, 1994; Stitt et al., Cell 80:661-670, 1995.

5.5.11. ANTI-INFLAMMATORY ACTIVITY

Proteins of the present invention may also exhibit anti-inflammatory activity. The anti-inflammatory activity may be achieved by providing a stimulus to cells involved in the inflammatory response, by inhibiting or promoting cell-cell interactions (such as, for example, cell adhesion), by inhibiting or promoting chemotaxis of cells involved in the inflammatory process, inhibiting or promoting cell extravasation, or by stimulating or suppressing production of other factors which more directly inhibit or promote an inflammatory response. Proteins exhibiting such activities can be used to treat inflammatory conditions including chronic or acute conditions), including without limitation intimation associated with infection (such as septic shock, sepsis or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine-induced lung injury, inflammatory bowel disease, Crohn's disease or resulting from over production of cytokines such as TNF or IL-1. Proteins of the invention may also be useful to treat anaphylaxis and hypersensitivity to an antigenic substance or material.

5.5.12. LEUKEMIAS

Leukemias and related disorders may be treated or prevented by administration of a therapeutic that promotes or inhibits function of the polynucleotides and/or polypeptides of the invention. Such leukemias and related disorders include but are not limited to acute

leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, chronic leukemia, chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia (for a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia).

5.5.13. NERVOUS SYSTEM DISORDERS

Nervous system disorders, involving cell types which can be tested for efficacy of intervention with compounds that modulate the activity of the polynucleotides and/or polypeptides of the invention, and which can be treated upon thus observing an indication of therapeutic utility, include but are not limited to nervous system injuries, and diseases or disorders which result in either a disconnection of axons, a diminution or degeneration of neurons, or demyelination. Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the invention include but are not limited to the following lesions of either the central (including spinal cord, brain) or peripheral nervous systems:

(i) traumatic lesions, including lesions caused by physical injury or associated with surgery, for example, lesions which sever a portion of the nervous system, or compression injuries;

(ii) ischemic lesions, in which a lack of oxygen in a portion of the nervous system results in neuronal injury or death, including cerebral infarction or ischemia, or spinal cord infarction or ischemia;

(iii) malignant lesions, in which a portion of the nervous system is destroyed or injured by malignant tissue which is either a nervous system associated malignancy or a malignancy derived from non-nervous system tissue;

(iv) infectious lesions, in which a portion of the nervous system is destroyed or injured as a result of infection, for example, by an abscess or associated with infection by human immunodeficiency virus, herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, syphilis;

(v) degenerative lesions, in which a portion of the nervous system is destroyed or injured as a result of a degenerative process including but not limited to degeneration associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis;

(vi) lesions associated with nutritional diseases or disorders, in which a portion of the nervous system is destroyed or injured by a nutritional disorder or disorder of metabolism including but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary degeneration of the corpus callosum), and alcoholic cerebellar degeneration;

(vii) neurological lesions associated with systemic diseases including but not limited to diabetes (diabetic neuropathy, Bell's palsy), systemic lupus erythematosus, carcinoma, or sarcoidosis;

(viii) lesions caused by toxic substances including alcohol, lead, or particular neurotoxins; and

(ix) demyelinated lesions in which a portion of the nervous system is destroyed or injured by a demyelinating disease including but not limited to multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy or various etiologies, progressive multifocal leukoencephalopathy, and central pontine myelinolysis.

Therapeutics which are useful according to the invention for treatment of a nervous system disorder may be selected by testing for biological activity in promoting the survival or differentiation of neurons. For example, and not by way of limitation, therapeutics which elicit any of the following effects may be useful according to the invention:

(i) increased survival time of neurons in culture;

(ii) increased sprouting of neurons in culture or in vivo;

(iii) increased production of a neuron-associated molecule in culture or in vivo, e.g., choline acetyltransferase or acetylcholinesterase with respect to motor neurons; or

(iv) decreased symptoms of neuron dysfunction in vivo.

Such effects may be measured by any method known in the art. In preferred, non-limiting embodiments, increased survival of neurons may be measured by the method set forth in

Arakawa et al. (1990, J. Neurosci. 10:3507-3515); increased sprouting of neurons may be detected by methods set forth in Pestronk et al. (1980, Exp. Neurol. 70:65-82) or Brown et al. (1981, Ann. Rev. Neurosci. 4:17-42); increased production of neuron-associated molecules may be measured by bioassay, enzymatic assay, antibody binding, Northern blot assay, etc., depending on the molecule to be measured; and motor neuron dysfunction may be measured by assessing the physical manifestation of motor neuron disorder, *e.g.*, weakness, motor neuron conduction velocity, or functional disability.

In a specific embodiments, motor neuron disorders that may be treated according to the invention include but are not limited to disorders such as infarction, infection, exposure to toxin, trauma, surgical damage, degenerative disease or malignancy that may affect motor neurons as well as other components of the nervous system, as well as disorders that selectively affect neurons such as amyotrophic lateral sclerosis, and including but not limited to progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio syndrome, and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

5.5.14. OTHER ACTIVITIES

A protein of the invention may also exhibit one or more of the following additional activities or effects: inhibiting the growth, infection or function of, or killing, infectious agents, including, without limitation, bacteria, viruses, fungi and other parasites; effecting (suppressing or enhancing) bodily characteristics, including, without limitation, height, weight, hair color, eye color, skin, fat to lean ratio or other tissue pigmentation, or organ or body part size or shape (such as, for example, breast augmentation or diminution, change in bone form or shape); effecting biorhythms or caricadic cycles or rhythms; effecting the fertility of male or female subjects; effecting the metabolism, catabolism, anabolism, processing, utilization, storage or elimination of dietary fat, lipid, protein, carbohydrate, vitamins, minerals, co-factors or other nutritional factors or component(s); effecting behavioral characteristics, including, without limitation, appetite, libido, stress, cognition

(including cognitive disorders), depression (including depressive disorders) and violent behaviors; providing analgesic effects or other pain reducing effects; promoting differentiation and growth of embryonic stem cells in lineages other than hematopoietic lineages; hormonal or endocrine activity; in the case of enzymes, correcting deficiencies of the enzyme and treating deficiency-related diseases; treatment of hyperproliferative disorders (such as, for example, psoriasis); immunoglobulin-like activity (such as, for example, the ability to bind antigens or complement); and the ability to act as an antigen in a vaccine composition to raise an immune response against such protein or another material or entity which is cross-reactive with such protein.

5.6. PHARMACEUTICAL FORMULATIONS AND ROUTES OF ADMINISTRATION

A protein of the present invention (from whatever source derived, including without limitation from recombinant and non-recombinant sources) may be administered to a patient in need, by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s) at doses to treat or ameliorate a variety of disorders. Such a composition may also contain (in addition to protein and a carrier) diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain other factors such as, but not limited to, M-CSF, GM-CSF, TNF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IFN, TNF0, TNF1, TNF2, G-CSF, Meg-CSF, thrombopoietin, stem cell factor, and erythropoietin. The pharmaceutical composition may further contain other agents which either enhance the activity of the protein or compliment its activity or use in treatment. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with protein of the invention, or to minimize side effects.

Conversely, protein of the present invention may be included in formulations of a particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent. A protein of the present invention may be active in multimers (e.g., heterodimers or homodimers) or complexes with itself or other proteins. As a result, pharmaceutical compositions of the invention may comprise a protein of the invention in such multimeric or complexed form.

Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms, e.g., treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. When applied to an individual active ingredient, administered alone, a therapeutically effective dose refers to that ingredient alone. When applied to a combination, a therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of protein of the present invention is administered to a mammal having a condition to be treated. Protein of the present invention may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, protein of the present invention may be administered either simultaneously with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering protein of the present invention in combination with cytokine(s),

lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

5.6.1. ROUTES OF ADMINISTRATION

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.Administration of protein of the present invention used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, topical application or cutaneous, subcutaneous, intraperitoneal, parenteral or intravenous injection. Intravenous administration to the patient is preferred.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a arthritic joints or in fibrotic tissue, often in a depot or sustained release formulation. In order to prevent the scarring process frequently occurring as complication of glaucoma surgery, the compounds may be administered topically, for example, as eye drops.Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with a specific antibody, targeting, for example, arthritic or fibrotic tissue. The liposomes will be targeted to and taken up selectively by the afflicted tissue.

5.6.2. COMPOSITIONS/FORMULATIONS

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. These pharmaceutical compositions may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.Proper formulation is dependent upon the route of administration chosen. When a therapeutically effective amount of protein of

the present invention is administered orally, protein of the present invention will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% protein of the present invention, and preferably from about 25 to 90% protein of the present invention. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of protein of the present invention, and preferably from about 1 to 50% protein of the present invention.

When a therapeutically effective amount of protein of the present invention is administered by intravenous, cutaneous or subcutaneous injection, protein of the present invention will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to protein of the present invention, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining

the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present

invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or

by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase

carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols. Many of the proteinase inhibiting compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Such pharmaceutically acceptable base addition salts are those salts which retain the biological effectiveness and properties of the free acids and which are obtained by reaction with inorganic or organic bases such as sodium hydroxide, magnesium hydroxide, ammonia, trialkylamine, dialkylamine, monoalkylamine, dibasic amino acids, sodium acetate, potassium benzoate, triethanol amine and the like.

The pharmaceutical composition of the invention may be in the form of a complex of the protein(s) of present invention along with protein or peptide antigens. The protein and/or peptide antigen will deliver a stimulatory signal to both B and T lymphocytes. B lymphocytes will respond to antigen through their surface immunoglobulin receptor. T lymphocytes will respond to antigen through the T cell receptor (TCR) following presentation of the antigen by MHC proteins. MHC and structurally related proteins including those encoded by class I and class II MHC genes on host cells will serve to present the peptide antigen(s) to T lymphocytes. The antigen components could also be supplied as purified MHC-peptide complexes alone or with co-stimulatory molecules that can directly signal T cells. Alternatively antibodies able to bind surface immunoglobulin and other molecules on B cells as well as antibodies able to bind the TCR and other molecules on T cells can be combined with the pharmaceutical composition of the invention. The pharmaceutical composition of the invention may be in the form of a liposome in which protein of the present invention is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. Nos. 4,235,°71; 4,501,728; 4,837,028; and

4,737,323, all of which are incorporated herein by reference.

The amount of protein of the present invention in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of protein of the present invention with which to treat each individual patient. Initially, the attending physician will administer low doses of protein of the present invention and observe the patient's response. Larger doses of protein of the present invention may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.01 μg to about 100 mg (preferably about 0.1 μg to about 10 mg, more preferably about 0.1 μg to about 1 mg) of protein of the present invention per kg body weight. For compositions of the present invention which are useful for bone, cartilage, tendon or ligament regeneration, the therapeutic method includes administering the composition topically, systematically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone, cartilage or tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than a protein of the invention which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the composition in the methods of the invention. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering the protein-containing composition to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular

application of the compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyglycolic acid and polyanhydrides. Other potential materials are biodegradable and biologically well-defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability. Presently preferred is a 50:50 (mole weight) copolymer of lactic acid and glycolic acid in the form of porous particles having diameters ranging from 150 to 800 microns. In some applications, it will be useful to utilize a sequestering agent, such as carboxymethyl cellulose or autologous blood clot, to prevent the protein compositions from disassociating from the matrix.

A preferred family of sequestering agents is cellulosic materials such as alkylcelluloses (including hydroxyalkylcelluloses), including methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropyl-methylcellulose, and carboxymethylcellulose, the most preferred being cationic salts of carboxymethylcellulose (CMC). Other preferred sequestering agents include hyaluronic acid, sodium alginate, poly(ethylene glycol), polyoxyethylene oxide, carboxyvinyl polymer and poly(vinyl alcohol). The amount of sequestering agent useful herein is 0.5-20 wt %, preferably 1-10 wt % based on total formulation weight, which represents the amount necessary to prevent desorbtion of the protein from the polymer matrix and to provide appropriate handling of the composition, yet not so much that the progenitor cells are prevented from infiltrating the matrix, thereby providing the protein the opportunity to assist the osteogenic activity of the progenitor cells. In further compositions, proteins of the invention may be combined with other agents beneficial to the treatment of the bone

and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factors (TGF-.alpha. and TGF-.beta.), and insulin-like growth factor (IGF).

The therapeutic compositions are also presently valuable for veterinary applications. Particularly domestic animals and thoroughbred horses, in addition to humans, are desired patients for such treatment with proteins of the present invention. The dosage regimen of a protein-containing pharmaceutical composition to be used in tissue regeneration will be determined by the attending physician considering various factors which modify the action of the proteins, e.g., amount of tissue weight desired to be formed, the site of damage, the condition of the damaged tissue, the size of a wound, type of damaged tissue (e.g., bone), the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and with inclusion of other proteins in the pharmaceutical composition. For example, the addition of other known growth factors, such as IGF I (insulin like growth factor I), to the final composition, may also effect the dosage. Progress can be monitored by periodic assessment of tissue/bone growth and/or repair, for example, X-rays, histomorphometric determinations and tetracycline labeling.

Polynucleotides of the present invention can also be used for gene therapy. Such polynucleotides can be introduced either in vivo or ex vivo into cells for expression in a mammalian subject. Polynucleotides of the invention may also be administered by other known methods for introduction of nucleic acid into a cell or organism (including, without limitation, in the form of viral vectors or naked DNA). Cells may also be cultured ex vivo in the presence of proteins of the present invention in order to proliferate or to produce a desired effect on or activity in such cells. Treated cells can then be introduced in vivo for therapeutic purposes.

5.6.3. EFFECTIVE DOSAGE

Pharmaceutical compositions suitable for use in the present invention include

compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of the C-proteinase activity). Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. *See, e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1.Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the C-proteinase inhibiting effects, or minimal effective

concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; for example, the concentration necessary to achieve 50-90% inhibition of the C-proteinase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

5.6.4. PACKAGING

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition.

5.7. ANTIBODIES

Another aspect of the invention is an antibody that specifically binds the polypeptide of the invention. Such antibodies can be either monoclonal or polyclonal antibodies, as well fragments thereof and humanized forms or fully human forms, such as those produced in transgenic animals. The invention further provides a hybridoma that produces an antibody according to the invention. Antibodies of the invention are useful

for detection and/or purification of the polypeptides of the invention.

Protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the protein. Such antibodies may be obtained using either the entire protein or fragments thereof as an immunogen. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Methods for synthesizing such peptides are known in the art, for example, as in R. P. Merrifield, J. Amer. Chem. Soc. 85, 2149-2154 (1963); J. L. Krstenansky, et al., FEBS Lett. 211, 10 (1987). Monoclonal antibodies binding to the protein of the invention may be useful diagnostic agents for the immunodetection of the protein. Neutralizing monoclonal antibodies binding to the protein may also be useful therapeutics for both conditions associated with the protein and also in the treatment of some forms of cancer where abnormal expression of the protein is involved. In the case of cancerous cells or leukemic cells, neutralizing monoclonal antibodies against the protein may be useful in detecting and preventing the metastatic spread of the cancerous cells, which may be mediated by the protein. In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (Campbell, A.M., *Monoclonal Antibodies Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth *et al., J. Immunol. 35*:1-21 (1990); Kohler and Milstein, *Nature 256*:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al., Immunology Today 4*:72 (1983); Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985), pp. 77-96).

Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with a peptide or polypeptide of the invention. Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of the protein encoded by the ORF of the present invention used for immunization will vary based on the animal which is immunized, the antigenicity of the peptide and the site of injection. The

protein that is used as an immunogen may be modified or administered in an adjuvant in order to increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as globulin or β-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells. Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz *et al., Exp. Cell Research. 175*:109-124 (1988)).

Hybridomas secreting the desired antibodies are cloned and the class and subclass is determined using procedures known in the art (Campbell, A.M., *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984)). Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to proteins of the present invention.

For polyclonal antibodies, antibody containing antiserum is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures. The present invention further provides the above-described antibodies in delectably labeled form. Antibodies can be delectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.) fluorescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, etc. Procedures for accomplishing such labeling are well-known in the art, for example, see (Sternberger, L.A. *et al., J. Histochem. Cytochem. 18*:315 (1970); Bayer, E.A. *et al., Meth. Enzym. 62*:308 (1979); Engval, E. *et al., Immunol. 109*:129 (1972); Goding, J.W. *J. Immunol. Meth. 13*:215 (1976)).

The labeled antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays to identify cells or tissues in which a fragment of the polypeptide of interest is expressed. The antibodies may also be used directly in therapies or other diagnostics.The present invention further provides the above-described antibodies immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir, D.M. *et al.*, *"Handbook of Experimental Immunology"* 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986); Jacoby, W.D. *et al., Meth. Enzym. 34* Academic Press, N.Y. (1974)). The immobilized antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays as well as for immuno-affinity purification of the proteins of the present invention.

5.8. COMPUTER READABLE SEQUENCES

In one application of this embodiment, a nucleotide sequence of the present invention can be recorded on computer readable media. As used herein, "computer readable media" refers to any medium which can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. A skilled artisan can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising computer readable medium having recorded thereon a nucleotide sequence of the present invention. As used herein, "recorded" refers to a process for storing information on computer readable medium. A skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate manufactures comprising the nucleotide sequence information of the present invention.

A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide sequence of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the nucleotide sequence information of the present invention on computer readable medium. The sequence information can be represented in a word processing text file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like. A skilled artisan can readily adapt any number of dataprocessor structuring formats (e.g. text file or database) in order to obtain computer readable medium having recorded thereon the nucleotide sequence information of the present invention. By providing the nucleotide sequence of SEQ ID NOS:1, 2 or nucleotides 261-641 of SEQ ID NO:2 or a representative fragment thereof, or a nucleotide sequence at least 99.9% identical to SEQ ID NOS:1, 2 or 5 in computer readable form, a skilled artisan can routinely access the sequence information for a variety of purposes. Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium. The examples which follow demonstrate how software which implements the BLAST (Altschul *et al., J. Mol. Biol. 215*:403-410 (1990)) and BLAZE (Brutlag *et al., Comp. Chem. 17*:203-207 (1993)) search algorithms on a Sybase system is used to identify open reading frames (ORFs) within a nucleic acid sequence. Such ORFs may be protein encoding fragments and may be useful in producing commercially important proteins such as enzymes used in fermentation reactions and in the production of commercially useful metabolites.

As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used to analyze the nucleotide sequence information of the present invention. The minimum hardware means of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled artisan can readily appreciate that any one of the

currently available computer-based systems are suitable for use in the present invention. As stated above, the computer-based systems of the present invention comprise a data storage means having stored therein a nucleotide sequence of the present invention and the necessary hardware means and software means for supporting and implementing a search means. As used herein, "data storage means" refers to memory which can store nucleotide sequence information of the present invention, or a memory access means which can access manufactures having recorded thereon the nucleotide sequence information of the present invention.

As used herein, "search means" refers to one or more programs which are implemented on the computer-based system to compare a target sequence or target structural motif with the sequence information stored within the data storage means. Search means are used to identify fragments or regions of a known sequence which match a particular target sequence or target motif. A variety of known algorithms are disclosed publicly and a variety of commercially available software for conducting search means are and can be used in the computer-based systems of the present invention. Examples of such software includes, but is not limited to, MacPattern (EMBL), BLASTN and BLASTA (NPOLYPEPTIDEIA). A skilled artisan can readily recognize that any one of the available algorithms or implementing software packages for conducting homology searches can be adapted for use in the present computer-based systems. As used herein, a "target sequence" can be any nucleic acid or amino acid sequence of six or more nucleotides or two or more amino acids. A skilled artisan can readily recognize that the longer a target sequence is, the less likely a target sequence will be present as a random occurrence in the database. The most preferred sequence length of a target sequence is from about 10 to 100 amino acids or from about 30 to 300 nucleotide residues. However, it is well recognized that searches for commercially important fragments, such as sequence fragments involved in gene expression and protein processing, may be of shorter length.

As used herein, "a target structural motif," or "target motif," refers to any rationally selected sequence or combination of sequences in which the sequence(s) are chosen based on a three-dimensional configuration which is formed upon the folding of the target motif.

There are a variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzyme active sites and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, hairpin structures and inducible expression elements (protein binding sequences).

5.9. TRIPLE HELIX FORMATION

In addition, the fragments of the present invention, as broadly described, can be used to control gene expression through triple helix formation or antisense DNA or RNA, both of which methods are based on the binding of a polynucleotide sequence to DNA or RNA. Polynucleotides suitable for use in these methods are usually 20 to 40 bases in length and are designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee *et al., Nucl. Acids Res. 6*:3073 (1979); Cooney *et al., Science 15241*:456 (1988); and Dervan *et al., Science 251*:1360 (1991)) or to the mRNA itself (antisense - Olmno, *J. Neurochem. 56*:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988)). Triple helix- formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques have been demonstrated to be effective in model systems. Information contained in the sequences of the present invention is necessary for the design of an antisense or triple helix oligonucleotide.

5.10. DIAGNOSTIC ASSAYS AND KITS

The present invention further provides methods to identify the presence or expression of one of the ORFs of the present invention, or homolog thereof, in a test sample, using a nucleic acid probe or antibodies of the present invention.

In general, methods for detecting a polynucleotide of the invention can comprise contacting a sample with a compound that binds to and forms a complex with the polynucleotide for a period sufficient to form the complex; and detecting the complex, so that if a complex is detected, a polynucleotide of the invention is detected in the sample.

Such methods can also comprise contacting a sample under stringent hybridization conditions with nucleic acid primers that anneal to a polynucleotide of the invention under such conditions, and amplifying annealed polynucleotides, so that if a polynucleotide is amplified, a polynucleotide of the invention is detected in the sample.

In general, methods for detecting a polypeptide of the invention can comprise contacting a sample with a compound that binds to and forms a complex with the polypeptide for a period sufficient to form the complex, and detecting the complex, so that if a complex is detected, a polypeptide of the invention is detected in the sample. In detail, such methods comprise incubating a test sample with one or more of the antibodies or one or more of nucleic acid probes of the present invention and assaying for binding of the nucleic acid probes or antibodies to components within the test sample.

Conditions for incubating a nucleic acid probe or antibody with a test sample vary. Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the nucleic acid probe or antibody used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or immunological assay formats can readily be adapted to employ the nucleic acid probes or antibodies of the present invention. Examples of such assays can be found in Chard, T., *An Introduction to Radioimmunoassay and Related Techniques*, Elsevier Science Publishers, Amsterdam, The Netherlands (1986); Bullock, G.R. *et al., Techniques in Immunocytochemistry*, Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., *Practice and Theory of immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1985). The test samples of the present invention include cells, protein or membrane extracts of cells, or biological fluids such as sputum, blood, serum, plasma, or urine. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is compatible with the system utilized.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the assays of the present invention. Specifically, the invention provides a compartment kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the probes or antibodies of the present invention; and (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of a bound probe or antibody.

In detail, a compartment kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the antibodies used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound antibody or probe. Types of detection reagents include labeled nucleic acid probes, labeled secondary antibodies, or in the alternative, if the primary antibody is labeled, the enzymatic, or antibody binding reagents which are capable of reacting with the labeled antibody. One skilled in the art will readily recognize that the disclosed probes and antibodies of the present invention can be readily incorporated into one of the established kit formats which are well known in the art.

5.11. SCREENING ASSAYS

Using the isolated proteins and polynucleotides of the invention, the present invention further provides methods of obtaining and identifying agents which bind to a polypeptide encoded by the ORF from a polynucleotide with a sequence of SEQ ID NOS: 1, 2, nucleotides 261-641 of SEQ ID NO: 2, or to a specific domain of the polypeptide encoded by the nucleic acid, or to a nucleic acid with a sequence of SEQ ID NOS: 1, 2, or

nucleotides 261-641 of SEQ ID NO: 2. In detail, said method comprises the steps of:

(a) contacting an agent with an isolated protein encoded by an ORF of the present invention, or nucleic acid of the invention; and

(b) determining whether the agent binds to said protein or said nucleic acid.

In general, therefore, such methods for identifying compounds that bind to a polynucleotide of the invention can comprise contacting a compound with a polynucleotide of the invention for a time sufficient to form a polynucleotide/compound complex, and detecting the complex, so that if a polynucleotide/compound complex is detected, a compound that binds to a polynucleotide of the invention is identified.

Likewise, in general, therefore, such methods for identifying compounds that bind to a polypeptide of the invention can comprise contacting a compound with a polypeptide of the invention for a time sufficient to form a polypeptide/compound complex, and detecting the complex, so that if a polypeptide/compound complex is detected, a compound that binds to a polynucleotide of the invention is identified.

Methods for identifying compounds that bind to a polypeptide of the invention can also comprise contacting a compound with a polypeptide of the invention in a cell for a time sufficient to form a polypeptide/compound complex, wherein the complex drives expression of a receptor gene sequence in the cell, and detecting the complex by detecting reporter gene sequence expression, so that if a polypeptide/compound complex is detected, a compound that binds a polypeptide of the invention is identified.

Compounds identified via such methods can include compounds which modulate the activity of a polypeptide of the invention (that is, increase or decrease its activity, relative to activity observed in the absence of the compound). Alternatively, compounds identified via such methods can include compounds which modulate the expression of a polynucleotide of the invention (that is, increase or decrease expression relative to expression levels observed in the absence of the compound). Compounds, such as compounds identified via the methods of the invention, can be tested using standard assays well known to those of skill in the art for their ability to modulate activity/expression.

The agents screened in the above assay can be, but are not limited to, peptides,

carbohydrates, vitamin derivatives, or other pharmaceutical agents. The agents can be selected and screened at random or rationally selected or designed using protein modeling techniques.

For random screening, agents such as peptides, carbohydrates, pharmaceutical agents and the like are selected at random and are assayed for their ability to bind to the protein encoded by the ORF of the present invention. Alternatively, agents may be rationally selected or designed. As used herein, an agent is said to be "rationally selected or designed" when the agent is chosen based on the configuration of the particular protein. For example, one skilled in the art can readily adapt currently available procedures to generate peptides, pharmaceutical agents and the like capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides, for example see Hurby *et al.*, Application of Synthetic Peptides: Antisense Peptides," In *Synthetic Peptides, A User's Guide*, W.H. Freeman, NY (1992), pp. 289-307, and Kaspczak *et al.*, *Biochemistry 28*:9230-8 (1989), or pharmaceutical agents, or the like.

In addition to the foregoing, one class of agents of the present invention, as broadly described, can be used to control gene expression through binding to one of the ORFs or EMFs of the present invention. As described above, such agents can be randomly screened or rationally designed/selected. Targeting the ORF or EMF allows a skilled artisan to design sequence specific or element specific agents, modulating the expression of either a single ORF or multiple ORFs which rely on the same EMF for expression control. One class of DNA binding agents are agents which contain base residues which hybridize or form a triple helix formation by binding to DNA or RNA. Such agents can be based on the classic phosphodiester, ribonucleic acid backbone, or can be a variety of sulfhydryl or polymeric derivatives which have base attachment capacity.

Agents suitable for use in these methods usually contain 20 to 40 bases and are designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee *et al.*, *Nucl. Acids Res. 6*:3073 (1979); Cooney *et al.*, *Science 241*:456 (1988); and Dervan *et al.*, *Science 251*:1360 (1991)) or to the mRNA itself (antisense - Okano, *J. Neurochem. 56*:560 (1991); *Oligodeoxymucleotides as Antisense Inhibitors of*

Gene Expression, CRC Press, Boca Raton, FL (1988)). Triple helix- formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques have been demonstrated to be effective in model systems. Information contained in the sequences of the present invention is necessary for the design of an antisense or triple helix oligonucleotide and other DNA binding agents. Agents which bind to a protein encoded by one of the ORFs of the present invention can be used as a diagnostic agent, in the control of bacterial infection by modulating the activity of the protein encoded by the ORF. Agents which bind to a protein encoded by one of the ORFs of the present invention can be formulated using known techniques to generate a pharmaceutical composition.

5.12. USE OF NUCLEIC ACIDS AS PROBES

Another aspect of the subject invention is to provide for polypeptide-specific nucleic acid hybridization probes capable of hybridizing with naturally occurring nucleotide sequences. The hybridization probes of the subject invention may be derived from the nucleotide sequence of the SEQ ID NOS:1 or 2. Because the corresponding gene is only expressed in a limited number of tissues, especially adult tissues, a hybridization probe derived from SEQ ID NOS:1 or 2 can be used as an indicator of the presence of RNA of cell type of such a tissue in a sample.

Any suitable hybridization technique can be employed, such as, for example, in situ hybridization.PCR as described US Patent Nos 4,683,195 and 4,965,188 provides additional uses for oligonucleotides based upon the nucleotide sequences. Such probes used in PCR may be of recombinant origin, may be chemically synthesized, or a mixture of both. The probe will comprise a discrete nucleotide sequence for the detection of identical sequences or a degenerate pool of possible sequences for identification of closely related genomic sequences.

Other means for producing specific hybridization probes for nucleic acids include the cloning of nucleic acid sequences into vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to

synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate radioactively labeled nucleotides. The nucleotide sequences may be used to construct hybridization probes for mapping their respective genomic sequences. The nucleotide sequence provided herein may be mapped to a chromosome or specific regions of a chromosome using well known genetic and/or chromosomal mapping techniques. These techniques include in situ hybridization, linkage analysis against known chromosomal markers, hybridization screening with libraries or flow-sorted chromosomal preparations specific to known chromosomes, and the like. The technique of fluorescent in situ hybridization of chromosome spreads has been described, among other places, in Verma et al (1988). Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York NY.

Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques may be correlated with additional genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265:1981f). Correlation between the location of a nucleic acid on a physical chromosomal map and a specific disease (or predisposition to a specific disease) may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier or affected individuals.The nucleotide sequence may be used to produce purified polypeptides using well known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Goeddel (1990) Gene Expression Technology, Methods and Enzymology, Vol 185, Academic Press, San Diego. Polypeptides may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which a particular polypeptide nucleotide sequence was isolated or from a different species. Advantages of producing polypeptides by recombinant DNA technology include obtaining adequate amounts of the protein for purification and the availability of simplified purification procedures.

Each sequence so obtained was compared to sequences in GenBank using a search

algorithm developed by Applied Biosystems and incorporated into the INHERIT™ 670 Sequence Analysis System. In this algorithm, Pattern Specification Language (developed by TRW Inc., Los Angeles, CA) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search. Peptide and protein sequence homologies were ascertained using the INHERIT™ 670 Sequence Analysis System in a way similar to that used in DNA sequence homologies. Pattern Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology that were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

Alternatively, BLAST, which stands for Basic Local Alignment Search Tool, is used to search for local sequence alignments (Altschul SF (1993) J Mol Evol 36:290-300; Altschul, SF et al (1990) J Mol Biol 215:403-10). BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. Whereas it is ideal for matches which do not contain gaps, it is inappropriate for performing motif-style searching. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP). An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches.

E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

In addition, BLAST analysis was used to search for related molecules within the libraries of the LIFESEQ™ database. This process, an "electronic northern" analysis is analogous to northern blot analysis in that it uses one cellubrevin sequence at a time to search for identical or homologous molecules at a set stringency. The stringency of the electronic northern is based on "product score". The product score is defined as (% nucleotide or amino acid [between the query and reference sequences] in Blast multiplied by the % maximum possible BLAST score [based on the lengths of query and reference sequences]) divided by 100. At a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Homologous or related molecules can be identified by selecting those which show product scores between approximately 15 and 30.

The present invention is illustrated in the following examples. Upon consideration of the present disclosure, one of skill in the art will appreciate that many other embodiments and variations may be made in the scope of the present invention. Accordingly, it is intended that the broader aspects of the present invention not be limited to the disclosure of the following examples.

6.0. INDUSTRIAL APPLICABILITY:

6.1 EXAMPLE 1

Novel Chemokines Obtained from a cDNA Library of Fetal Liver-Spleen

A plurality of novel nucleic acids were obtained from the b^2HFLS20W cDNA library prepared from human fetal liver-spleen tissue, as described in Bonaldo et al., Genome Res. 6:791-806 (1996), using standard pcr, SBH sequence signature analysis and Sanger sequencing techniques. The inserts of the library were amplified with pcr using primers specific for vector sequences which flank the inserts. These samples were spotted onto nylon membranes and interrogated with oligonucleotide probes to give sequence signatures. The

clones were clustered into groups of similar or identical sequences, and single representative clones were selected from each group for gel sequencing. The 5' sequence of the amplified inserts was then deduced using the reverse M13 sequencing primer in a typical Sanger sequencing protocol. PCR products were purified and subjected to flourescent dye terminator cycle sequencing. Single pass gel sequencing was done using a 377 Applied Biosystems (ABI) sequencer. Two (2) of these inserts have been identifed as novel sequences not previously obtained from this library, and not previously reported in public databases. These sequences are shown in Figure 2 as SEQ ID NO. 1 and 2. The polypeptide sequences corresponding to these nucleic acid sequences are shown in Figure 3 as SEQ ID NO. 3. These amino acid sequences have striking homology to the chemokines of the C-X-C, C-C and C subfamilies of chemokines.

6.2 EXAMPLE 2

USE OF SEQ ID NO. 2

The novel chemokine polypeptides of the invention are useful in medical imaging, e.g., imaging the site of infection, inflammation, and other sites having chemokine receptor molecules. See, e.g., Kunkel et al., U.S. Pat. No. 5,413,778. Such methods involve chemical attachment of a labelling agent, administration of the labelled chemokine to a subject in a pharmaceutically acceptable carrier, ,and imaging the labelled chemokine *in vivo* at the target site.

The myelosuppressive activity of the novel chemokine polypeptides of the invention are assayed by injection of the novel chemokine polypeptides into mice, e.g., as described by Maze et al., J. Immunol., 149:1004-1009 (1992) for the measurement of the myelosuppressive action of MIP-1 alpha . A single dose of 0.2 to 10 ug of recombinant polypeptide is intravenously injected into C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Me.). The myelosuppressive effect of the novel chemokine is then measured by the cycling rates of myeloid progenitor cells in the femoral bone marrow and spleen.

Molecules which bind SEQ ID No. 3 will include, for e.g., monoclonal antibodies and and other small molecules which act as blocking agents, or as activators. *See* above.

These molecules are identified as agonists or antagonists of SEQ ID No. 3 by the following types of assays. In preferred embodiments, the binding molecules will be antagonists which cause cell death.

Agonists and antagonists of chemotaxis are identified in a transmigration assay, e.g., leukocyte cells to be tested are added to the upper chamber of a transwell containing polycarbonate membranes, 8.0 um pore size (Costar, Cambridge, Mass.). Novel chemokine polypeptide and the agonist or antagonist are added to the lower chamber of the transwell at various concentrations. At the end of the assay, cells that have transmigrated through the membrane into the lower chamber are collected and counted. Agonists and antagonists are identified by differences in chemotaxis from transwells using the novel chemokine alone.

Agonist and antagonists of the novel chemokine polypeptides may be identified by standard *in vitro* assays by measuring the inhibition of GM colony (CFU-GM) and cluster formation. Such assays are well known and representative assays are described in Gentile et al., U.S. Pat. Nos. 5,149,544 and 5,294,544. In these assays, bone marrow or spleen cells are stimulated with, e.g., CSF, in an in vitro culture system. The activity of the agonist or antagonist on the novel chemokine polypeptide is measured as the amount it changes the CSF-stimulated colony and cluster formation in the presence of the novel chemokine polypeptide alone.

Agonists and antagonists of the novel chemokine polypeptides activity with monocytes/macrophages or human neutrophils are identified by, e.g., methods described by Devi et al., J. Immunol., 153:5376-5383 (1995). Indices of activation measured in such studies include increased adhesion to fibrinogen due to integrin activation, chemotaxis, induction of reactive nitrogen intermediates, respiratory burst (superoxide and hydrogen peroxide production), and exocytosis of lysozyme and elastase in the presence of cytochalasin B. As discussed by Devi et al., these activities correlate to several stages of the leukocyte response to inflammation. This leukocyte response, reviewed by Springer, Cell, 76:301-314 (1994), involves adherence of leukocytes to endothelial cells of blood vessels, migration through the endothelial layer, chemotaxis toward a source of chemokines, and site-specific release of inflammatory mediators. Agonists and antagonists of any one of these

stages provide an important tools for clinical intervention, and for modulation of the inflammatory response.

Antibodies or other suitable binding molecules which bind to SEQ ID No. 3 are also useful in receptor protein purification and for *in situ* hybridization analyses. Initial *in situ* analyses identify associations between the expression of SEQ ID No. 3 and genetic disorders of the immune system or development. *In situ* hybridization with these binding molecules then diagnoses these genetic disorders of the immune system or development in potential patients.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and compositions and methods which are functionally equivalent are within the scope of the invention. Indeed, numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the present preferred embodiments. Consequently, the only limitations which should be placed upon the scope of the invention are those which appear in the appended claims.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising:

a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:3;

b) nucleotide sequence of SEQ ID NO:2;

c) nucleotides 261-641 of the nucleotide sequence of SEQ ID NO:2;

d) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence encoded by the cDNA insert of clone p12616HY;

e) a nucleotide sequence comprising the full length protein coding sequence of the cDNA insert of clone p12616HY;

f) a nucleotide sequence encoding the mature protein encoded by the cDNA insert of clone p12616HY.; or

g) the nucleotide sequence of the cDNA insert of clone p12616HY.

2. An isolated polynucleotide which hybridizes to the complement of the polynucleotide of Claim 1 under stringent hybridization conditions.

3. An isolated polynucleotide which comprises the complement of the polynucleotide of Claim 1.

4. A vector comprising the isolated polynucleotide of Claim 1 or 2.

5. An expression vector comprising the isolated polynucleotide of Claim 1 or 2.

6. A host cell genetically engineered to contain the polynucleotide of Claim 1 or 2.

7. A host cell genetically engineered to contain the polynucleotide of Claim 1 or 2 in operative association with a regulatory sequence that controls expression of the polynucleotide in the host cell.

8. An isolated polypeptide comprising:

a) the amino acid sequence of SEQ ID NO:3;

b) the amino acid sequence encoded by the cDNA insert of clone p12616HY;

c) an amino acid sequence comprising the full length protein encoded by the cDNA insert of clone p12616HY; or

d) a polypeptide comprising the mature protein encoded by the cDNA insert of clone p12616HY.

9. A composition comprising the polypeptide of Claim 8 and a carrier.

10. An antibody directed against the polypeptide of Claim 8.

11. A method for detecting a polynucleotide of Claim 1 or 2 in a sample, comprising:

a) contacting the sample with a compound that binds to and forms a complex with the polynucleotide for a period sufficient to form the complex; and

b) detecting the complex,

so that if a complex is detected, a polynucleotide of Claim 1 or 2 is detected.

12. A method for detecting a polynucleotide of Claim 1 or 2 in a sample, comprising:

a) contacting the sample under stringent hybridization conditions with

nucleic acid primers that anneal to a polynucleotide of Claim 1 or 2 under such conditions; and

b) amplifying the annealed polynucleotides,

so that if a polynucleotide is amplified, a polynucleotide of Claim 1 or 2 is detected.

13. The method of Claim 12, wherein the polynucleotide is an RNA molecule that encodes a polypeptide of Claim 8, and the method further comprises reverse transcribing an annealed RNA molecule into a cDNA polynucleotide.

14. A method for detecting a polypeptide of Claim 8 in a sample, comprising:

a) contacting the sample with a compound that binds to and forms a complex with the polypeptide for a period sufficient to form the complex; and

b) detecting the complex,

so that if a complex is detected, a polypeptide of Claim 8 is detected.

15. A method for identifying a compound that binds to a polypeptide of Claim 8, comprising:

a) contacting a compound with a polypeptide of Claim 8 for a time sufficient to form a polypeptide/compound complex; and

b) detecting the complex,

so that if a polypeptide/compound complex is detected, a compound that binds to a polypeptide of Claim 8 is identified.

16. A method for identifying a compound that binds to a polypeptide of Claim 8, comprising:

a) contacting a compound with a polypeptide of Claim 8, in a cell, for

a time sufficient to form a polypeptide/compound complex, wherein the complex drives expression of a reporter gene sequence in the cell; and

b) detecting the complex by detecting reporter gene sequence expression,

so that if a polypeptide/compound complex is detected, a compound that binds to a polypeptide of Claim 8 is identified.

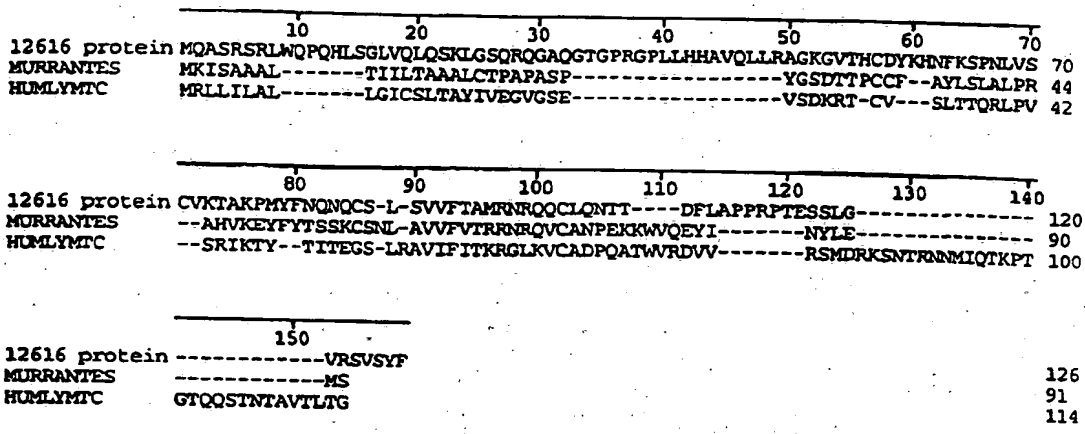
17. A method of modulating activity of a polypeptide of Claim 8, comprising contacting a cell that expresses the polypeptide with a compound that modulates activity of the polypeptide for a time sufficient to modulate said activity.

18. A method of modulating activity of the polypeptide of Claim 8, comprising contacting the polypeptide with a compound that modulates activity of the polypeptide for a time sufficient to modulate said activity.

19. A method of producing the polypeptide of claim 8, comprising,

a) culturing the host cell of claim 7 for a period of time sufficient to express the polypetide contained within said cell; and

b) isolating the peptide from the cell of step (a).



12616 protein: Translated 12616 nucleotide sequence
MURRANTES: Mouse RANTES
HUMLYMTC: Human lymphotactin

FIG. 1

TACGAATTTAAAACGACTCACTATMCGGAATTTGGCCCTCGAGGCCAAGAATTCGGCACGAGGATTGAAGAA
GCTGGTTTATTAGAACCAGCCTGTCGCTTTTCAAAGCTGCTTAAAAATAAGATACTGACCTCACCCCTAGAG
ATGATTCAGTGGCCCTGGGTGGGGCCAAGAAATCCGTGGTGTTTTGTAAGCACTGCTGCCCATTTCTCATGG
CAGTAAAGACCACACTGAGACTGCACTGATTTTGATTAAAGTACATAGGCTTGGCTGTTTTTACACAGCTAA
CTAAATTTGGACTCTTAAAATTGTGTTTGTAATCACAGTGTGTTACACCTTTCCCAGCTCTCAGCAGTTGTA
CTGCATGATGGAGCAGCGGGCCCCTGGGACCTGTTCCCTGTGCTCCCTGCCTCTGAGAGCCCAGCTTGCTCT
GAAGTTGGACTAAACCACTTAGATGCTGGGGTTGCCACAGCCTGCTCCTGGAAGCCTGCATTCCAGTCAGGT
TGCATCCTTCTGGTGCTGACAGAACCCAGTGGTGAAGCCAAAGCACAGGAATGCTTTAAAAATGAACAGTTT
ATGCAGAATAAGGGTCAGGAGTCATGCCAGACCAGAACTTGATGCTACTGTGTCTTGTGTTAAGAATCATTT
CCTGGCCAGGCTCGGTGGCTCAGGCCTGTAATCCCAGCACTTTGGGAGGCTGAGGCAGGTGAATCACTTGGT
CAGGAGATGGAGACCATTCTGGCTAACATGGTG

(SEQ ID No. 1)

CACCATGTTAGCCAGAATGGTCTCCATCTCCTGACCAAGTGATTCACCTGCCTCAGCCTCCCAAAGTGCTGG
GATTACAGGCCTGAGCCACCGAGCCTGGCCAGGAAATGATTCTTAACACAAGACACAGTAGCATCAAGTTCT
GGTCTGGCATGACTCCTGACCCTTATTCTGCATAAACTGTTCATTTTTAAAGCATTCCTGTGCTTTGGCTTC
ACCACTGGGTTCTGTCAGCACCAGAAGGATGCAACCTGACTGGAATGCAGGCTTCCAGGAGCAGGCTGTGGC
AACCCCAGCATCTAAGTGGTTTAGTCCAACTTCAGAGCAAGCTGGGCTCTCAGAGGCAGCGAGCACAGGAA
CAGGTCCCAGGGGCCCGCTGCTCCATCATGCAGTACAACTGCTGAGAGCTGGGAAAGGTGTAACACACTGTG
ATTACAAACACAATTTTAAGAGTCCAAATTTAGTTAGCTGTGTAAAAACAGCCAAGCCTATGTACTTTAATC
AAAATCAGTGCAGTCTCAGTGTGGTCTTTACTGCCATGAGAAATCGCCAGCAGTGCTTACAAAACACCACGG
ATTTCTTGGCCCCACCCAGGCCCACTGAATCATCTCTAGGGGTGAGGTCAGTATCTTATTTTTAAGCAGCTT
TGAAAAGCGACAGGCTGGTTCTAATAAACCAGCTTCTTCAATCCTCGTGCCGAATTCTTGGCCTCGAGGGCC
AAATTCCGKATAGTGAGTCGTTTTAAATTCGTA

(SEQ ID No. 2)

FIG. 2

MQASRSRLWQPQHLSGLVQLQSKLGSQRQGAQGTGPRGPLLHHAVQLLRAGKGVTHCDYKHNFKSPNLVSCV
KTAKPMYFNQNQCSLSVVFTAMRNRQQCLQNTTDFLAPPRPTESSLGVRSVSYF
(SEQ ID No. 3)

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/26236

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C07K14/52 G01N33/53 G01N33/68 C12Q1/68
C07K16/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MILLER M.D. ET AL.: "Biology and Biochemistry of the Chemokines: a family of chemotactic and inflammatory cytokines" CRITICAL REVIEWS IN IMMUNOLOGY, vol. 12, no. 1,2, 1992, pages 17-46, XP002050850	
A	WO 97 35027 A (HUMAN GENOME SCIENCES INC (US); NI J.; LI H.; SU J.Y.) 25 September 1997 see abstract see page 58 - page 60; claims	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 1999	06/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/26236

Box I Obs rvations where certain laims w re found uns archable (Continuati n of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 17 and 18, as far as in vivo methods are concerned, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.